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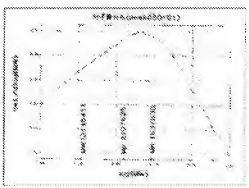
(54) SALT-TOLERANT BACILLUS SUBTILIS VAR. CHUNGKOOKJANG STRAIN PRODUCING HIGH MOLECULAR WEIGHT POLY-γ-GLUTAMIC ACID

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a method for efficiently producing high molecular weight poly-γ-glutamic acid with a Bacillus strain.
SOLUTION: The Bacillus Subtilis var. chungkookjang strain (KCTC0697BP) separated from chungkookjang. The method for producing the poly-γ-glutamic acid with the strain. The poly-γ-glutamic acid produced with the strain and having a molecular weight of ≥2,000 kDa.



CLAIMS

[Claim(s)]
[Claim 1]Produce Polly gamma-glutamic acid which is a biodegradable polymer substance, and by salt tolerance. A bacillus which sporulation was difficult, and did not contain a plasmid in the strain itself, but was separated from a natto fermented soybeans and bean paste hot pot (******) Subtilis A natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).
[Claim 2]A bacillus whose deposition number nitrate reduction power is KCTC0697BP in negativity in Claim 1 Subtilis A natto fermented soybeans and bean paste hot pot stock (Bacillus subtilisvar.chungkookjang).
[Claim 3]A recombination protein production method using the microorganism according to claim 1 or 2 as a host.
[Claim 4]A manufacturing method of Polly gamma-glutamic acid using the microorganism according to claim 1 or 2.
[Claim 5]A manufacturing method of Polly gamma-glutamic acid including the following stage in Claim 4. (a) Polly gamma-glutamic-acid content liquid which carried out the stage (b) above-mentioned acquisition which cultivates a microorganism Claim 1 or given in two, and acquires Polly gamma-glutamic acid removes polysaccharide, A stage condensed after carrying out stage (d) dialysis into which processed to protease and extracellular nature protein was made to disassemble after dissolving stage (c) above-mentioned Polly gamma-glutamic-acid precipitate which acquires a Polly gamma-glutamic-acid sediment next it carried out solvent extraction and centrifuged and removing isolation glutamic acid [Claim 6]Polly gamma-glutamic acid which is manufactured by the microorganism according to claim 1 or 2, and is characterized by a molecular weight being 2,000 or more kDa.
[Claim 7]Cosmetics containing the Polly gamma-glutamic acid according to claim 6.
[Claim 8]Health food containing the Polly gamma-glutamic acid according to claim 6.
[Claim 9]A drink containing the Polly gamma-glutamic acid according to claim 6.
[Claim 10]Drugs containing the Polly gamma-glutamic acid according to claim 6.

[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention straw. The salt-tolerant bacillus Subtilis (bacillus subtlis) natto fermented soybeans and bean paste hot pot stock separated from the natto fermented soybeans and bean paste hot pot (******) which is a tradition beans fermented food of used South Korea (KCTC Bacillus subtilis var.chungkookjang) It is related with Polly gamma-glutamic acid which is an extracellular secretion nature polymer produced from 0697BP and said strain, and is edible, water solubility, negative ion nature, and a biodegradable polymer substance. The D-amino acid transaminase which is an enzyme in which this invention makes keto acid transfer the amino group of D-amino acid to details more (D-amino acid aminotransferase:EC2.6.1.21). (It is hereafter called D-AAT for short). The nature object formation of the opposite sex of an alanine and glutamic acid. Glutamic-acid racemase (Glutamate racemase:EC5.1.1.3: call for short the following GluRA) and alanine racemase (Alanine racemase: call for short the following AlaRA) which are enzymes which carry out a catalyst, And it is related with the Polly gamma-glutamic acid produced by the new strain which produces Polly gamma-glutamic acid out of a cell with intracellular enzyme complexes, such as a Polly gamma-glutamate synthesis enzyme (Poly-gamma-glutamate synthetase), and said strain. As illustrated to drawing 1, many enzymes are participating in composition of Polly gamma-glutamic acid.
[0002]
[Description of the Prior Art]Polly gamma-glutamic acid is the polymer which carried out Poliy gamma-glutamyl (gamma-glutamiyl) combination, and D and L-glutamic acid as mucous material, It is produced from the genus Bacillus stock separated from "KINEMA" etc. which are the "natto fermented soybeans and bean paste hot pot" (******) which is a tradition beans fermented food of South Korea using straw, the "fermented soybeans" which are Japanese tradition beans fermented foods, and a tradition beans fermented food of Nepal. The Polly gamma-glutamic acid produced from said genus Bacillus stock Edible, It can use for the raw material substance for the natural decomposition nature plastic manufacture by the desiccant, the moisturizer and the raw material of cosmetics, and the affinity of an ester derivative by water solubility, negative ion nature, and a biodegradable polymer substance (molecular weight: 100,000-2,000,000).
[0003]Recently, the research which got interested in production of Polly gamma-glutamic acid, development and the soluble fiber of a heat-resistant plastic, film production according to the substitutive-goods raw material of a difficulty degradable polymer and an esterification reaction about use, etc. has been advancing actively mainly by industrialized nations. The development of hydro-gel (hydrogel) and industrialization research by the change-in-physical-properties research and the crosslinking bond agent which are caused in Polly gamma-glutamic acid at the time of gamma irradiation are promoted. The influence of manganese ion which it will have on the presentation of Polly gamma-glutamic acid, and Polly gamma-glutamic-acid production if an example is given, The research to use to a water-soluble polymer and research (Biosci.Biotechnol.Biochem., 60(8):1239-1242-1996) on development of the low-water-flow solubility plastic by composition of an ester derivative, and bacillus by ultrasonic decomposition. The practical use (JP,H6-32742,A) to health food with the ********** curative effect as the Polly gamma-glutamic-acid production by Subtilis and a calcium resolvent, etc. see.
[0004]In addition, the effect (Euro patent No. 838160) of decreasing the phosphorus content of a drainage system and decreasing water pollution, Biodegradable adsorbent resin with the high gelation properties by radiation irradiation and absorptivity is manufactured, and there is a report of application (JP,H10-251402,A), practical use (JP,H7-300522,A, JP,H6-322358,A), etc. to sanitary goods, foodstuffs, and horticulture industry of a diaper etc. the use (JP,H7-138364,A.) as the solidification biodegradable fiber by the dissolution of Polly gamma-glutamic acid, precipitate, and desiccation, a film, and a film formation agent There is also a report to JP,H5-117388,A, polymer for drug carriers (JP,H6-92870,A, JP,H6-256220,A), etc.
[0005]On the other hand, in South Korea with fundamental researches, such as efficient production (South

Korean patent application No. 3404 [1997 to], South Korean patent application No. 67605 [1997 to]), a characteristic improvement, etc. of Polly gamma-glutamic acid. The application study which is going to use for the source material of cosmetics the Polly gamma-glutamic acid which a bacillus Bacillus natto stock produces by the Pacific Ocean, Inc. occurs.

[0006]

[Problem(s) to be Solved by the Invention]However, the molecular weights of the Polly gamma **GURUTAMIN acid obtained by the method using the conventional genus Bacillus stock are 100,000-2,000,000, and for a desiccant, a moisturizer, or natural decomposition nature plastic manufacture, The method that productivity was higher was called for the direction which produces the Polly gamma **GURUTAMIN acid of Polymer Division more.

[0007]Therefore, this invention aims to let a molecular weight provide the method of producing more 2,000,000 or more Polly gamma **GURUTAMIN acid to a large quantity using a genus Bacillus stock.

[0008]

[Means for Solving the Problem]Salt-tolerant strain bacillus separated from a natto fermented soybeans and bean paste hot pot (******) of a South Korean tradition beans fermented food as a result of this invention persons' inquiring wholeheartedly to achieve the above objects Subtilis A natto fermented soybeans and bean paste hot pot stock, It finds out producing Polly gamma-glutamic acid of the amount of Polymer Division at high concentration, and came to complete this invention based on these knowledge.

[0009]Namely, this invention Polly gamma-glutamic acid which is a biodegradable polymer substance is produced, and it is salt tolerance, Bacillus which sporulation was difficult, and did not contain a plasmid in the strain itself, but was separated from a natto fermented soybeans and bean paste hot pot (******) Subtilis It is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).

[0010]Bacillus whose nitrate reduction power the above-mentioned strain is netative in the above-mentioned invention and whose deposition number is KCTC0697BP Subtilis It is preferred that it is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).

[0011]Another of an invention is a recombination protein production method using the above-mentioned strain as a host.

[0012]Furthermore, another of an invention is a manufacturing method of Polly gamma-glutamic acid using the above-mentioned strain.

[0013]In the above-mentioned invention, it is preferred to include the following stage.

(a) Polly gamma-glutamic-acid content liquid which carried out the stage (b) above-mentioned acquisition which cultivates the above-mentioned strain and acquires Polly gamma-glutamic acid removes polysaccharide, A stage condensed after carrying out stage (d) dialysis into which processed to protease and extracellular nature protein was made to disassemble after dissolving stage (c) above-mentioned Polly gamma-glutamic-acid precipitate which acquires a Polly gamma-glutamic-acid sediment next it carried out solvent extraction and centrifuged and removing isolation glutamic acid [0014]According to this invention, how a molecular weight produces efficiently 2,000,000 or more Polly gamma **GURUTAMIN acid can be provided.

[0015]Furthermore, another of an invention is Polly gamma-glutamic acid which is manufactured by the above-mentioned strain and characterized by a molecular weight being 2,000 or more kDa.

[0016]According to this invention, the Polly gamma **GURUTAMIN acid which fitted a desiccant, a moisturizer, and natural decomposition nature plastic manufacture rather than the Polly gamma **GURUTAMIN acid produced by the conventional genus Bacillus stock can be provided.

[0017]Furthermore, another of an invention is the cosmetics containing the above-mentioned Polly gamma-glutamic acid.

[0018]Furthermore, another of an invention is the health food containing the above-mentioned Polly gamma-glutamic acid.

[0019]Furthermore, another of an invention is a drink containing the above-mentioned Polly gamma-glutamic acid.

[0020]Furthermore, another of an invention is the drugs containing the above-mentioned Polly gamma-glutamic acid.

[0021]According to this invention, cosmetics, health food, a drink, drugs, etc. which contain Polly gamma-glutamic acid of the amount of Polymer Division conventionally can be provided.

[0022]

[Embodiment of the Invention]Hereafter, this invention is explained more concretely.

[0023](Separation and identification of a strain) Bacillus which is a new strain of this invention which produced Polly gamma-glutamic acid of edible, water solubility, negative ion nature, and biodegradability with high yield, and had salt tolerance Subtilis Separation of a natto fermented soybeans and bean paste hot pot and the method of

identification are as follows.
[0024]It is produced in the Republic of Korea, and in order to separate a strain with a Polly gamma-glutamic-acid high throughput from the sample of 20 kinds of ****** which are a tradition beans fermented food using straw, various ****** samples are heat-treated for 20 minutes with a 60 ** constant temperature bath, after being suspended to distilled water. the colony pure isolation of the bacillus which cultivates for three days with 37 ** humidistat, and expresses high viscosity after carrying out the smear of said suspension small quantity made to heat-treat to the Polly gamma-glutamic-acid production agar plate culture medium (GS) containing 1.5% of L-glutamic acid -- it carries out. After carrying out subculture twice for these separation bacillus repeatedly using the same above double grounds, a strain with the most active biomass growth is separated in the bacillus colony from which high viscosity is taken out by production of Polly gamma-glutamic acid. Although said separated Polly gamma-glutamic-acid high throughput strain forms a milky bacillus colony from LB plate agar which contains agar 2%, this is cultivated at 37 ** by a **** thin method for 20 hours, and the strain it becomes active [growth of a biomass] most [strain] is separated.
[0025]this invention strain separated by the above-mentioned method is morphological, and the physiological character is as follows.
[0026]When cultivating by LB agar plate culture medium, opalescence carries out bacillus colony formation of this invention strain, and it has the characteristic that biomass growth becomes slow in the culture temperature of not less than 55 ** as the gram positive bacteria with active growth of a biomass on not less than 37 ** golden opportunity conditions. Bacillus with the common this invention strain It is a salt-tolerant strain producible also by 9.0% of salt (NaCl) concentration higher than the salt tolerance concentration which Subtilis has, the result of having made the comparative analysis of the 16S rDNA base rank of this invention separation strain to the strain 16S rDNA base sequence in a bacillus conventionally -- the homology (99.0%) of bacillus Subtilis (Bacillus subtilis) and very high 16S rDNA base sequence -- a table -- the bottom.
[0027]However, in spite of the above high homology, this invention -- bacillus in a new separation bacillus Subtilis . A natto fermented soybeans and bean paste hot pot can be used also for the strain which suited the high manifestation system of the recombination protein which did not contain the plasmid unlike the **** genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production, and let gene manipulation pass. The separation strain by this invention is a safe microorganism in which edible is possible. Therefore, for example, a vaccine can be made to be able to reveal by the ability to make said strain into a host (making the antigen portion of for example, a pig diarrhea virus reveal), and the strain itself can be used for the feed additives for a diarrhea disease therapy or prevention.
[0028]That is, oral vaccine development is attained using the strain of this invention.
[0029]Unlike other genus Bacillus stocks, nitrate reduction power is netative, sporulation does not happen easily and the separation strain of this invention has the characteristic which is not easily derived by manganese ion, either. It is a bacillus about the strain of the aforementioned result to this invention. It classifies into Subtilis and is a bacillus. Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) is named, The name of said strain was made into 'Bacillus BS-4' (Bacillus sp.BS-4) for convenience, and it ****ed to the Biotechnology Division research institute gene bank (KCTC. Taejon Metropolitan ********** 52 whereabouts) as an accession number of KCTC0697BP on November 18, 1999.
[0030](Analysis of Polly gamma-glutamic acid and activity measurement of an intervention enzyme) A fixed quantity of the Polly gamma-glutamic acid produced by said strain, D of a polymer, and the check of an L-glutamic acid presentation are carried out as follows.
[0031]Bacillus Subtilis After cultivating a natto fermented soybeans and bean paste hot pot, liquid, such as an upper group which centrifuged the culture medium and Polly gamma-glutamic acid contained, is separated, and D and L-glutamic acid are separated using the crepuscular-rays study activity HPLC column which added high concentration chloride here and was hydrolyzed at the elevated temperature. In order to ask for a standard curve, the refined Polly gamma-glutamic-acid sample was also analyzed by the same method. The content of the Polly gamma-glutamic acid which calculated the correction value over the isolation L-glutamic acid which carried out semi- [of the substance which passed the column] to D and an L-glutamic acid standard curve, and was added to the initial culture medium quality and after quantifying, and was produced purely is calculated.
[0032]For measurement of intracellular enzyme D-AAT and GluRA which participate in production of biodegradable Polly gamma-glutamic acid directly, and AlaRA activity, After collecting biomasses and crushing a biomass by an ultrasonic crusher next it inoculated this invention strain into 5-ml LB liquid medium and 10 hours cultivated at 37 **, it centrifuges and crude enzyme liquid is obtained. An activity fixed quantity of D-AAT makes the crude enzyme liquid obtained by the above react it to D-alanine and alpha-keto glutamic acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and by an enzyme reaction. The activity can be quantified by measuring the quantity of the pyruvic acid which is a reaction product produced, GluRA can analyze the L-

glutamic acid produced by the post-enzyme reaction which made crude enzyme liquid react to D-glutamic acid, alpha-glutamine acid, and PLP into 50mM tris buffer solution (Tris-HCl, pH 8.5) in an optical activity HPLC column, and can make the activity a fixed quantity. AlaRA measures with an absorbance the pyruvic acid produced with alanine dehydrogenase by making D-alanine into a disposition at 340 nm, and makes the activity a fixed quantity.

[0033]Hereafter, this invention is explained more to details through working example. It is for these working example only explaining this invention more concretely, and the range of this invention is not limited by these working example according to the gist of this invention.

[0034]

[Example](Working example 1) ****** which is the tradition beans fermented food produced by the traditional beans bacterial coupling through the decomposition of a microorganism and the separation straw of an identification 1, microorganism which produce Polly gamma-glutamic acid came to hand all over the country, and it was used as a sample. After following the endospore formation-ized process which is heat-treated for 20 minutes with a 60 ** constant temperature bath, and a bacillus has next it added each sample to sterilization distilled water in small quantities and was suspended. The smear of the suspension is carried out to GS plate agar (1.5% L-glutamic acid, 5.0% sugar, 0.27%KH_2PO_4, 0.42%Na_2HPO_4, 0.05%NaCl, and 0.05%$MgSO_4$ and 0.05% BIOKEN) which contains agar 2%. It cultivated for three days to a 37 ** incubator. The biomass which forms the mucosity bacillus colony shown by Polly gamma-glutamic-acid production was separated after culture.

[0035]A possibility that a separation bacillus can be co-cultured by mucosity Polymer Division Polly gamma-glutamic-acid production is taken into consideration. After carrying out the smear according to a continuation thin method on LB culture medium which is a general culture medium which does not produce a polymer. biomass growth carried out pure isolation only of the most flourishing bacillus, and selected with the Polly gamma-glutamic-acid production strain, and biochemical characterization was examined closely using that said strain is morphological and a general culture medium without polymer production of mucosity.

[0036]In order to investigate the constitutive enzyme activity of the enzyme complex which participates in polymer production of the Polly gamma-glutamic-acid production strain obtained by this invention, the separated strain was inoculated into 5-ml LB liquid medium, and 10 hours cultivated it. Said culture medium was centrifuged, biomasses were collected, and after crushing the biomass by the ultrasonic crusher and obtaining crude enzyme liquid, this was used for D-AAT and GluRA which participate in Polly gamma-glutamic-acid production, and AlaRA enzyme activity measurement.

[0037]2, and morphological and it is biochemical characterization (1) . [a microorganism]   Although edible [which was separated in growth and the gestalt characteristic above-mentioned stage of   microorganism], water solubility, biodegradability, and the activity strain from ** ionicity Polly gamma-glutamic-acid Takao formed the colony of the bacillus which takes out high mucosity with GS agar plate background which is a Polly gamma-glutamic-acid production culture medium. By LB agar plate culture medium which does not produce mucosity pile composition, the milky bacillus colony was formed and many of biomasses showed the cylindrical gestalt. Under the temperature influence which it has on biomass growth, growth was nursed in [not less than 30 **] 55 ** or less, and biomass growth was not able to be checked above 60 **.

[0038]When microscope observation was carried out by the exponential phase using LB liquid medium of biomass growth, it is a bacillus of a comparison strain. It was similar with Subtilis (graphic display abbreviation). It was a Gram positive and the sizes of the cell were 0.7 to 0.8X2.0-3.0 micrometers of outlines. RICHINIPOMISU which is other comparison strains expressed the cylindrical pattern that the biomass outside of an exponential phase was thin on the other hand again, and the separation strain of this invention expressed the different biomass outside.

[0039](2) The strain and bacillus by sodium chloride tolerance this invention After 24 hours cultivated the Subtilis fermented-soybeans (B. subtilis natto) strain by LB culture medium by which NaCl of each concentration was added, the absorbance was measured at 660 nm and the growth grade of each strain was measured (drawing 2). From the density range (12%) where the strain by this invention can grow so that it may see by a diagram to a bacillus Compared with the Subtilis fermented soybeans, it turns out that it is the tolerance over a twice [about] as many survival rate, i.e., sodium chloride, as this.

[0040](3) The strain by plasmid content characteristic this invention, bacillus Bacillus Subtilis 168 and the bacillus which are the type strains of Subtilis The plasmid was separated from Subtilis fermented-soybeans IFO3336, and the content propriety was checked (drawing 3). At drawing 3, M is 1kb rudder marker and A is a bacillus. Subtilis 168, the strain according [B] to this invention, and C express bacillus Subtilis fermented-soybeans IFO3336.

[0041]Although the strain by this invention is a strain which produces Polly gamma-glutamic acid as it sees by a diagram, it turns out that a plasmid like fermented-soybeans IFO3336 is not contained. And the same feature as bacillus Subtilis 168 which is a strain which cannot produce Polly gamma-glutamic acid is seen.
[0042]The strain by this invention can be used also for the host who suited the high manifestation system (secretory production) of the recombination protein which did not contain a plasmid, therefore carried out gene manipulation like [at the time of explaining in full detail].
[0043](4) The spore was dyed and observed, after inoculating the strain by this invention into LB culture medium by which $CoSO_4$ of sporulation characteristic 2mM was added and cultivating for four days at 37 ** (graphic display abbreviation). Bacillus which is the Polly gamma-glutamic-acid production strain with same strain by this invention It has checked that sporogenous ability power had come out notably compared with the Subtilis fermented soybeans.
[0044](5) In addition, the biochemical characterization of the strain by this invention, etc. were investigated using biochemistry characteristic API50CHB and an API20E kit.
[0045]The strains by this invention are gram positive bacteria, do not have the reducing power of a nitrate and do not produce Indore. Gelatin and starch are decomposed, beta **GURIKOSHIDAZE and **-galactosidase are produced, and oxidase is produced. An urease can be produced and it can grow up altogether on golden opportunity base conditions. It expressed as what can use glycerol, galactose, glucose, a shook sirloin, malt sugar, and starch.
[0046]It is as [detailed / it having been morphological and having expressed biochemical characterization to Table 1] the microorganism sorted out by this invention.
[0047]
[Table 1]

特性	本菌切の菌株
グラム染色	陽性
形態	桿状
胞子形成	少し膨張（よく観察できない）
胞子の形成	0.7～0.8μ x 2.5～3.0μm
成長温度	20～55℃
pH9．7での成長	陽性
NaCl 10%での成長	陽性
好気条件下での成長	陽性
嫌気条件下での成長	陽性
運動性試験	陽性
硝酸塩還元	陰性
インドール形成	陰性
オキシダーゼ生成	陽性
カタラーゼ生成	陽性
ウレアーゼ生成	陰性
βガラクトシダーゼ生成	陰性

[0048](6) In order to identify more correctly the separation strain obtained by base sequence analysis this invention, gene base sequence analysis of 16S rDNA was carried out.
[0049]First, after amplifying 16S rDNA gene in PCR using N-end primer (5'-AGAGTTTGATCCTGGCTCAG-3') and C-end primer (5'-AGAAAGGAGGTGATCCAGCC-3'). Cloning was carried out to plasmid pT7Blue, and the whole base sequence was determined. It is a bacillus as a result of comparing much 16SrDNA base sequences and homology of a microorganism to whom 16S rDNA base sequence of the microorganism sorted out is reported conventionally. Homology was expressed as Subtilis 99.0% and it has judged as what is located in a system which was illustrated by drawing 4.
[0050](7) The separation strain of this invention shows the characteristic which does not contain a plasmid unlike the usual genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production in spite of identification of the separated strain, however homology high as mentioned above. Such the characteristic shows that the strain by this invention can use gene manipulation for the host who suited the high manifestation system of the recombination protein which led. Unlike a genus Bacillus stock, nitrate reduction power is netative, sporulation is not performed easily but the separation strain of this invention has the characteristic which is not easily derived to manganese ion.
[0051]It is a bacillus natto fermented soybeans and bean paste hot pot (Bacillus.) about the strain of this invention about the characteristic (following working example can explain) of the Polly gamma-glutamic acid

which the characteristic and this strain of the above strain itself produce. It classifies into the new strain belonging to Subtilis, and is a bacillus. Subtilis It is named subtilis var.chungkookjang, The name of said strain was made into Bacillus BS-4' (Bacillus sp.BS-4) for convenience, it ****ed to the Biotechnology Division research institute gene bank (KCTC, South Korean Taejon Metropolitan ********** 52 whereabouts) on November 18, 1999, and deposition number KCTC0697BP was given.

[0052](Working example 2) After it inoculated the separation strain of generation this invention of Polly gamma-glutamic acid into the Polly gamma-glutamic-acid production culture medium and 72 hours cultivated at 37 **, the Polly gamma-glutamic-acid content sample solution was acquired by adjusting so that the 2N solution of hydrochloric acid may be added and pH may be set to 3.0. 10 hours made said sample solution settle at 4 **, and polysaccharide in fermented mash was removed, it added so that it might become fermented mash twice the volume of said there about ethanol, and it fully mixed. After 10 hours made mixed liquor settle at 4 **, it centrifuged and the Polly gamma-glutamic-acid sediment was obtained. Add distilled water to said sediment and it was made to dissolve in it, protease was added so that it might be set to 100 ug(s)/ml, and 37 ** humidistat was made to decompose the quality of extracellular protein of 6 hours which carries out a between settlement reaction and exists in a Polly gamma-glutamic-acid sample. It condensed, after removing the glutamic acid which dialyzed and separated this with sufficient quantity of distilled water, and pure Polly gamma-glutamic acid was obtained. The these-refined Polly gamma-glutamic acid measured the presentation and the quantity of production of D and L glutamic acid which were obtained by performing oxidized water decomposition.

[0053]As the productivity of the Polly gamma-glutamic acid which the strain of this invention and the strain used for comparison produce was expressed to Table 2, as for the separation strain of this invention, the liquid medium showed the productivity of 16 g/L. Bacillus separated from fermented soybeans Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a showed the Polly gamma-glutamic-acid productivity of 10 g/L and 9 g/L respectively. In order to compare the productivity of Polly gamma-glutamic acid in a solid medium, After inoculating the bacillus into the plate agar which is a Polly gamma-glutamic-acid production culture medium which contains agar 2% and cultivating for three days at 37 **, Polly gamma-glutamic acid was refined identically to the above-mentioned refining method, and the difference of the productivity of this invention separation strain and a comparison strain was investigated. As for the test result and the separation strain of this invention, 8 mg / plate agar, and RICHIEPOMISU ATCC9945a expressed the productivity of 6 mg / plate agar, and 12 mg / plate agar, and bacillus Subtilis fermented-soybeans IFO3336a checked that this invention separation strain had twice [about] as many productivity as this compared with a comparison strain. The result of having measured the quantity of the Polly gamma-glutamic acid respectively produced per 0.3-mg strain so that it might see with the gel photograph of drawing 5, Bacillus which is a Polly gamma-glutamic-acid production strain of existing [the separation strain of this invention] It can check producing Polly gamma-glutamic acid of very much quantity from Subtilis fermented-soybeans IFO3336a,

[0054]

[Table 2]

[0055](Working example 3) The quantity of production of the Polly gamma-glutamic acid which D of Polly gamma-glutamic acid and the separation strain of stereospecificity investigation this invention of L-glutamic acid produce, and the presentation of D which is a constituent of Polly gamma-glutamic acid, and L-glutamic acid were investigated.

[0056]In order to investigate the percentage of D which is a monomer of Polly gamma-glutamic acid of the amount of Polymer Division which the separation strain of this invention produces, and L-glutamic acid, After making the pure Polly gamma-glutamic-acid sample which 72 hours cultivates with 150 rpm and 37 ** humidistat using the Erlenmeyer flask which is 500 ml which GS production culture medium contained, and could be refined in the above-mentioned refining method and the similar way add and deaerate 6N chloride, 10 hours hydrolyzed at 105 **.

[0057]The amino acid analysis of the above-mentioned hydrolysate uses the concentration gradient using 50mM phosphoric acid buffer solution (pH 7.0) which contains methanol 5%, and methanol. The HPLC column (RexchromeS5-100-ODS, Regis Chem, 4.6mmX25cmX5m, U.S.) analyzed. After separation of the stereoisomeric form made D and the amino-terminus part of L-glutamic acid derivatize using o-phthalaldehyde, In 452 nm (Em) and 342 nm (Ex), D and L-glutamic acid which are the constituents of Polly gamma-glutamic acid were made a fixed quantity according to the standard curve of D and L-glutamic acid with the fluorescence detector.

[0058]As the result of having investigated the content of D which is a monomer which constitutes the produced Polly gamma-glutamic acid, and L-glutamic acid was expressed to Table 2, The ratios of D/L-glutamic acid from the Poliy gamma-glutamic acid which this invention separation strain produces are about 40/60, Bacillus which is a comparison strain In Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a, the ratio of D/L-glutamic acid is 50/50, and the separation bacillus was able to see different monomer percentage.
[0059](Fixed quantity of enzyme activity which participates in Polly gamma-glutamic-acid production)
In order to measure the enzyme activity which participates in Polly gamma-glutamic-acid production of this invention separation strain, It centrifuged, after cultivating a biomass with 37 ** humidistat using LB liquid medium which does not produce a mucosity polymerization agent, and after adjusting crude enzyme liquid with the method which mentioned this above next, the enzyme activity included in crude enzyme liquid was measured.

[0060]It makes the activity of D-AAT react crude enzyme liquid to D-alanine and **-ketoglutaric acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and it by an enzyme reaction. It quantifies by measuring the quantity of the pyruvic acid which is the produced reaction product (Berntsson S, Anal.Chem., 27:1659-1660-1995), The activity of GluRA analyzed and quantified the L-glutamic acid produced by the enzyme reaction in the optical activity HPLC column, after making crude enzyme liquid react to D-glutamic acid, **-ketoglutaric acid, and PLP in 50mM tris buffer solution (Tris-HCl, pH 8.5). The spectrometry of the pyruvic acid which made alanine dehydrogenase react to the L-alanine produced considering D-alanine as a substrate, and was generated was carried out, and alanine racemase activity measurement (Biochemistry, 25:3261-3267,1986) quantified it. Protein content was measured by the Bradford method (Bradford, M., Anal.Biochem., 72:248-254-1976).
[0061]The activity measurement result of the quantity of Polly gamma-glutamic acid, a molecular weight and D, an L-glutamic acid ratio, and an enzyme (D-AAT, GluRA, AlaRA) by which the product from happiness in the next life is carried out of having cultivated the separation strain of this invention with the Erlenmeyer flask was shown in Table 2 and Table 3. Bacillus known as a Polly gamma-glutamic-acid production strain separated from Japanese fermented soybeans in order to compare the characteristic of the Polly gamma-glutamic acid which this invention separation strain produces Subtilis, and the Polly gamma-glutamic-acid quantity of production and enzyme activity of RICHIEPOMISU were measured and displayed.
[0062]
[Table 3]

菌株	酵素比活性 (mU/mg タンパク質)		
	D-AAT	GluRA	AlaRA
枯草菌168	0.003	0.0109	0.193
納豆菌 IFO3336a	0.186	0.0546	0.108
バチルス・リチエポミス ATCC9945a	0.167	0.0021	0.080

[0063]As a result of comparing and examining the above enzyme activity, the separation strain of this invention AlaRA, D-Ala and D-Glu of cell growth and Polly gamma-glutamic acid required for production are compounded using GluRA activity, Bacillus It can expect using the activity of D-AAT higher about 3 times than the Subtilis fermented soybeans and RICHIEPOMISU as a thing with the course which compounds D-Glu in large quantities and uses it for production of Polly gamma-glutamic acid directly from D-Ala, Bacillus The Subtilis fermented soybeans and RICHIEPOMISU so that Tables 2 and 3 and drawing 1 may see, It can expect as a thing with the course which compounds glutamic acid required for composition of cell growth and Polly gamma-glutamic acid using high GluRA activity, The separation strain of this invention is a bacillus. It was considered Subtilis fermented-soybeans IFO3336a and RICHIEPOMISU ATCC9945a as a thing with each-other different **** amino-acid-synthesis course (drawing 6). Are drawing 6 and the glutamine:2-oxo guru TAREDO amino mutase and 2 : A glutamine synthetase, 3 -- L-glutamic acid: -- as for the pyruvic acid amino mutase and 4, the D-amino acid amino mutase and 6 are Polly gamma-glutamate synthesis enzymes alanine racemase and 5, and TCA expresses a tricarboxylic acid cycle.
[0064]Namely, bacillus While D-glutamic acid which can be used for composition of Polly gamma-glutamic acid is converted into intracellular in the case of the Subtilis Bacillus natto stock, L-glutamic acid is converted into D-glutamic acid by operation of glutamic-acid racemase and it is made, In the separation strain of this invention, D-glutamic acid is produced from L-glutamic acid by operation of alanine racemase and the D-amino acid amino mutase.
[0065](Working example 4) bacillus which are a separation strain of determination-of-molecular-weight this invention by comparison (1) electrical-and-electric-equipment **** of the molecular weight of Polly gamma-glutamic acid, and the type strain in a bacillus Subtilis 168 -- and,Bacillus which is a comparison strain In order

to measure the molecular weight of the Polly gamma-glutamic acid which Subtilis fermented-soybeans IFO3336 produces, concentration gradient SDS-PAGE was carried out.

[0066]After refining the Polly gamma-glutamic acid produced from each biomass with the refining method explained in full detail in said working example 2, the about 200 ug(s)/ml solution was prepared. After mixing each Polly gamma-glutamic-acid solution 80ul with 5X buffer solution 20ul by which dyeing medicine was added, electric **** was performed by 5 to 20% of concentration gradient polyacrylamide gel. Standard protein and Polly gamma-glutamic acid were dyed for the electric **** completion back of each by a KOMASHI dyeing reagent and methylene blue (drawing 5). At drawing 5, M is standard protein and 1 is a bacillus. The strain according [according to / in Subtilis 168 and 2 / bacillus Subtilis fermented-soybeans IFO3336 / 3] to this invention was expressed.

[0067]Like drawing 5, the separation strain of this invention is a bacillus. It was able to check producing Polly gamma-glutamic acid of a far larger molecular weight than the molecular weight (about 1,000 KDa(s) - 2,000KDa) of the Polly gamma-glutamic acid which the Subtilis fermented soybeans produce.

[0068](2) After cultivating the separation strain of determination-of-molecular-weight this invention by a gel filtration chromatograph (GPC) for five days by GS solid medium, Polly gamma-glutamic acid was refined by the aforementioned method, and the molecular weight was analyzed using the gel penetration chromatograph (Asahipak GS-620 H+Tosoh TSK gel).

[0069]a gel filtration chromatograph -- 50mM salt: -- to the solvent, the rate of flow of the solvent carried out the acetonitrile (4:1) solution with 25 ** column oven at 0.7 ml/m. In the standard substance, polyethylene oxide was used and the molecular weight of Polly gamma-glutamic acid was measured using the refraction index measuring instrument.

[0070]The chromatograph of the test result was illustrated to drawing 7. As a result of analyzing this, as for the Polly gamma-glutamic acid which the separation strain by this invention produces, it turns out that Mw (an average molecular weight, weightaverage molecular weight) is [about 13 million and a molecular-weight-distribution figure (polydispersity)] about 8.0.

[0071]This proves that not only the chisel with a very large molecular weight compared with the thing which other strains produce but its molecular weight distribution of the Polly gamma-glutamic acid which the strain by this invention produces is uniform. Therefore, the Polly gamma-glutamic acid produced from the strain of this invention can be utilized very useful as an object for hydration gel manufacture.

[0072](Working example 5) Polly gamma-glutamic-acid molecular weight change of the strain by this invention which utilized Polly gamma-glutamic-acid decomposition activity measurement GPC of the strain by this invention, and followed culture time progress was investigated.

[0073]Cultivating the separation strain of this invention by GS solid medium, Polly gamma-glutamic acid was respectively refined by the aforementioned method on 1, 3, and the 5th at the time of progress, and a molecular weight and molecular weight distribution were investigated using GPC (Table 4).

[0074]

[Table 4]

表[illegible]	[illegible]	[illegible]
1	1.[illegible]x10^6	7.[illegible]
3	1.[illegible]x10^6	7.[illegible]
5	1.[illegible]x10^6	8.[illegible]

[0075]It turns out that the Polly gamma-glutamic acid compounded by the strain by this invention hardly changes an average molecular weight and molecular weight distribution even if culture time passes so that it may see in Table 4. therefore, strain bacillus by this invention Subtilis or [that a natto fermented soybeans and bean paste hot pot does not have Polly gamma-glutamic-acid decomposition activity] -- or it can be judged that there is not almost it.

[0076]

[Effect of the Invention]As it explains in detail by the above and being explained,Salt-tolerant strain bacillus which separated this invention from "******" (natto fermented soybeans and bean paste hot pot) which is a South Korean tradition beans fermented food Subtilis natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang, KCTC0697BP). And Polly gamma-glutamic acid which is edible, the water solubility, the negative ion nature, and the biodegradable polymer substance which are produced from said strain is provided. Bacillus of this invention Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) produces Polly gamma-glutamic acid with a larger molecular weight than Polly gamma-glutamic acid of the cell which a common genus Bacillus stock produces, The quantity of production is excellent again, and the Polly gamma-glutamic acid produced by the strain of this invention can be used for

product development, such as a high-value-added cosmetics raw material, a desiccant, and biodegradable plastic material, useful by composition and chemical preparation of a derivative.

[Translation done.]

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TECHNICAL FIELD

[Field of the Invention]This invention straw. The salt-tolerant bacillus Subtilis (bacillus subtlis) natto fermented soybeans and bean paste hot pot stock separated from the natto fermented soybeans and bean paste hot pot (******) which is a tradition beans fermented food of used South Korea (KCTC Bacillus subtilis var.chungkookjang) It is related with Polly gamma-glutamic acid which is an extracellular secretion nature polymer produced from 0697BP and said strain, and is edible, water solubility, negative ion nature, and a biodegradable polymer substance. The D-amino acid transaminase which is an enzyme in which this invention makes keto acid transfer the amino group of D-amino acid to details more (D-amino acid aminotransferase:EC2.6.1.21). (It is hereafter called D-AAT for short), The nature object formation of the opposite sex of an alanine and glutamic acid. Glutamic-acid racemase (Glutamate racemase:EC5.1.1.3; call for short the following GluRA) and alanine racemase (Alanine racemase; call for short the following AlaRA) which are enzymes which carry out a catalyst, And it is related with the Polly gamma-glutamic acid produced by the new strain which produces Polly gamma-glutamic acid out of a cell with intracelilular enzyme complexes, such as a Polly gamma-glutamate synthesis enzyme (Poly-gamma-glutamate synthetase), and said strain. As illustrated to drawing 1, many enzymes are participating in composition of Polly gamma-glutamic acid.

[Translation done.]

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PRIOR ART

[Description of the Prior Art]Polly gamma-glutamic acid is the polymer which carried out Polly gamma-glutamyl (gamma-glutamiyl) combination, and D and L-glutamic acid as mucous material, It is produced from the genus Bacillus stock separated from "KINEMA" etc. which are the "natto fermented soybeans and bean paste hot pot" (******) which is a tradition beans fermented food of South Korea using straw, the "fermented soybeans" which are Japanese tradition beans fermented foods, and a tradition beans fermented food of Nepal. The Polly gamma-glutamic acid produced from said genus Bacillus stock Edible, It can use for the raw material substance for the natural decomposition nature plastic manufacture by the desiccant, the moisturizer and the raw material of cosmetics, and the affinity of an ester derivative by water solubility, negative ion nature, and a biodegradable polymer substance (molecular weight: 100,000-2,000,000).
[0003]Recently, the research which got interested in production of Polly gamma-glutamic acid, development and the soluble fiber of a heat-resistant plastic, film production according to the substitutive-goods raw material of a difficulty degradable polymer and an esterification reaction about use, etc. has been advancing actively mainly by industrialized nations. The development of hydro-gel (hydrogel) and industrialization research by the change-in-physical-properties research and the crosslinking bond agent which are caused in Polly gamma-glutamic acid at the time of gamma irradiation are promoted. The influence of manganese ion which it will have on the presentation of Polly gamma-glutamic acid, and Polly gamma-glutamic-acid production if an example is given, The research to use to a water-soluble polymer and research (Biosci.Biotechnol.Biochem., 60(8):1239-1242-1996) on development of the low-water-flow solubility plastic by composition of an ester derivative, and bacillus by ultrasonic decomposition. The practical use (JP,H6-32742,A) to health food with the ********** curative effect as the Polly gamma-glutamic-acid production by Subtilis and a calcium resolvent, etc. see.
[0004]In addition, the effect (Euro patent No. 838160) of decreasing the phosphorus content of a drainage system and decreasing water pollution, Biodegradable adsorbent resin with the high gelation properties by radiation irradiation and absorptivity is manufactured, and there is a report of application (JP,H10-251402,A), practical use (JP,H7-300522,A, JP,H6-322358,A), etc. to sanitary goods, foodstuffs, and horticulture industry of a diaper etc. the use (JP,H7-138364,A.) as the solidification biodegradable fiber by the dissolution of Polly gamma-glutamic acid, precipitate, and desiccation, a film, and a film formation agent There is also a report to JP,H5-117388,A, polymer for drug carriers (JP,H6-92870,A, JP,H6-256220,A), etc.
[0005]On the other hand, in South Korea with fundamental researches, such as efficient production (South Korean patent application No. 3404 [1997 to], South Korean patent application No. 67605 [1997 to]), a characteristic improvement, etc. of Polly gamma-glutamic acid, The application study which is going to use for the source material of cosmetics the Polly gamma-glutamic acid which a bacillus Bacillus natto stock produces by the Pacific Ocean, Inc. occurs.

[Translation done.]

EFFECT OF THE INVENTION

[Effect of the Invention]As it explains in detail by the above and being explained,Salt-tolerant strain bacillus which separated this invention from "******" (natto fermented soybeans and bean paste hot pot) which is a South Korean tradition beans fermented food Subtilis natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang, KCTC0697BP). And Polly gamma-glutamic acid which is edible, the water solubility, the negative ion nature, and the biodegradable polymer substance which are produced from said strain is provided. Bacillus of this invention Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) produces Polly gamma-glutamic acid with a larger molecular weight than Polly gamma-glutamic acid of the cell which a common genus Bacillus stock produces, The quantity of production is excellent again, and the Polly gamma-glutamic acid produced by the strain of this invention can be used for product development, such as a high-value-added cosmetics raw material, a desiccant, and biodegradable plastic material, useful by composition and chemical preparation of a derivative.

[Translation done.]

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention]However, the molecular weights of the Polly gamma **GURUTAMIN acid obtained by the method using the conventional genus Bacillus stock are 100,000-2,000,000, and for a desiccant, a moisturizer, or natural decomposition nature plastic manufacture, The method that productivity was higher was called for the direction which produces the Polly gamma **GURUTAMIN acid of Polymer Division more.

[0007]Therefore, this invention aims to let a molecular weight provide the method of producing more 2,000,000 or more Polly gamma **GURUTAMIN acid to a large quantity using a genus Bacillus stock.

[Translation done.]

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MEANS

[Means for Solving the Problem]Salt-tolerant strain bacillus separated from a natto fermented soybeans and bean paste hot pot (******) of a South Korean tradition beans fermented food as a result of this invention persons' inquiring wholeheartedly to achieve the above objects Subtilis A natto fermented soybeans and bean paste hot pot stock, It finds out producing Polly gamma-glutamic acid of the amount of Polymer Division at high concentration, and came to complete this invention based on these knowledge.
[0009]Namely, this invention Polly gamma-glutamic acid which is a biodegradable polymer substance is produced, and it is salt tolerance, Bacillus which sporulation was difficult, and did not contain a plasmid in the strain itself, but was separated from a natto fermented soybeans and bean paste hot pot (******) Subtilis It is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).
[0010]Bacillus whose nitrate reduction power the above-mentioned strain is netative in the above-mentioned invention and whose deposition number is KCTC0697BP Subtilis It is preferred that it is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).
[0011]Another of an invention is a recombination protein production method using the above-mentioned strain as a host.
[0012]Furthermore, another of an invention is a manufacturing method of Polly gamma-glutamic acid using the above-mentioned strain.
[0013]In the above-mentioned invention, it is preferred to include the following stage.
(a) Polly gamma-glutamic-acid content liquid which carried out the stage (b) above-mentioned acquisition which cultivates the above-mentioned strain and acquires Polly gamma-glutamic acid removes polysaccharide, A stage condensed after carrying out stage (d) dialysis into which processed to protease and extracellular nature protein was made to disassemble after dissolving stage (c) above-mentioned Polly gamma-glutamic-acid precipitate which acquires a Polly gamma-glutamic-acid sediment next it carried out solvent extraction and centrifuged and removing isolation glutamic acid [0014]According to this invention, how a molecular weight produces efficiently 2,000,000 or more Polly gamma **GURUTAMIN acid can be provided.
[0015]Furthermore, another of an invention is Polly gamma-glutamic acid which is manufactured by the above-mentioned strain and characterized by a molecular weight being 2,000 or more kDa.
[0016]According to this invention, the Polly gamma **GURUTAMIN acid which fitted a desiccant, a moisturizer, and natural decomposition nature plastic manufacture rather than the Polly gamma **GURUTAMIN acid produced by the conventional genus Bacillus stock can be provided.
[0017]Furthermore, another of an invention is the cosmetics containing the above-mentioned Polly gamma-glutamic acid.
[0018]Furthermore, another of an invention is the health food containing the above-mentioned Polly gamma-glutamic acid.
[0019]Furthermore, another of an invention is a drink containing the above-mentioned Polly gamma-glutamic acid.
[0020]Furthermore, another of an invention is the drugs containing the above-mentioned Polly gamma-glutamic acid.
[0021]According to this invention, cosmetics, health food, a drink, drugs, etc. which contain Polly gamma-glutamic acid of the amount of Polymer Division conventionally can be provided.
[0022]
[Embodiment of the Invention]Hereafter, this invention is explained more concretely.
[0023](Separation and identification of a strain) Bacillus which is a new strain of this invention which produced Polly gamma-glutamic acid of edible, water solubility, negative ion nature, and biodegradability with high yield, and had salt tolerance Subtilis Separation of a natto fermented soybeans and bean paste hot pot and the method of identification are as follows.

[0024]It is produced in the Republic of Korea, and in order to separate a strain with a Polly gamma-glutamic-acid high throughput from the sample of 20 kinds of ****** which are a tradition beans fermented food using straw, various ****** samples are heat-treated for 20 minutes with a 60 ** constant temperature bath, after being suspended to distilled water, the colony pure isolation of the bacillus which cultivates for three days with 37 ** humidistat, and expresses high viscosity after carrying out the smear of said suspension small quantity made to heat-treat to the Polly gamma-glutamic-acid production agar plate culture medium (GS) containing 1.5% of L-glutamic acid -- it carries out. After carrying out subculture twice for these separation bacillus repeatedly using the same above double grounds, a strain with the most active biomass growth is separated in the bacillus colony from which high viscosity is taken out by production of Polly gamma-glutamic acid. Although said separated Polly gamma-glutamic-acid high throughput strain forms a milky bacillus colony from LB plate agar which contains agar 2%, this is cultivated at 37 ** by a **** thin method for 20 hours, and the strain it becomes active [growth of a biomass] most [strain] is separated.

[0025]this invention strain separated by the above-mentioned method is morphological, and the physiological character is as follows.

[0026]When cultivating by LB agar plate culture medium, opalescence carries out bacillus colony formation of this invention strain, and it has the characteristic that biomass growth becomes slow in the culture temperature of not less than 55 ** as the gram positive bacteria with active growth of a biomass on not less than 37 ** golden opportunity conditions. Bacillus with the common this invention strain It is a salt-tolerant strain producible also by 9.0% of salt (NaCl) concentration higher than the salt tolerance concentration which Subtilis has, the result of having made the comparative analysis of the 16S rDNA base rank of this invention separation strain to the strain 16S rDNA base sequence in a bacillus conventionally -- the homology (99.0%) of bacillus Subtilis (Bacillus subtilis) and very high 16S rDNA base sequence -- a table -- the bottom.

[0027]However, in spite of the above high homology, this invention -- bacillus in a new separation bacillus Subtilis . A natto fermented soybeans and bean paste hot pot can be used also for the strain which suited the high manifestation system of the recombination protein which did not contain the plasmid unlike the **** genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production, and let gene manipulation pass. The separation strain by this invention is a safe microorganism in which edible is possible. Therefore, for example, a vaccine can be made to be able to reveal by the ability to make said strain into a host (making the antigen portion of for example, a pig diarrhea virus reveal), and the strain itself can be used for the feed additives for a diarrhea disease therapy or prevention.

[0028]That is, oral vaccine development is attained using the strain of this invention.

[0029]Unlike other genus Bacillus stocks, nitrate reduction power is netative, sporulation does not happen easily and the separation strain of this invention has the characteristic which is not easily derived by manganese ion, either. It is a bacillus about the strain of the aforementioned result to this invention. It classifies into Subtilis and is a bacillus. Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) is named, The name of said strain was made into 'Bacillus BS-4' (Bacillus sp.BS-4) for convenience, and it ****ed to the Biotechnology Division research institute gene bank (KCTC, Taejon Metropolitan ********** 52 whereabouts) as an accession number of KCTC0697BP on November 18, 1999.

[0030](Analysis of Polly gamma-glutamic acid and activity measurement of an intervention enzyme) A fixed quantity of the Polly gamma-glutamic acid produced by said strain, D of a polymer, and the check of an L-glutamic acid presentation are carried out as follows.

[0031]Bacillus Subtilis After cultivating a natto fermented soybeans and bean paste hot pot, liquid, such as an upper group which centrifuged the culture medium and Polly gamma-glutamic acid contained, is separated, and D and L-glutamic acid are separated using the crepuscular-rays study activity HPLC column which added high concentration chloride here and was hydrolyzed at the elevated temperature. In order to ask for a standard curve, the refined Polly gamma-glutamic-acid sample was also analyzed by the same method. The content of the Polly gamma-glutamic acid which calculated the correction value over the isolation L-glutamic acid which carried out semi- [of the substance which passed the column] to D and an L-glutamic acid standard curve, and was added to the initial culture medium quality and after quantifying, and was produced purely is calculated.

[0032]For measurement of intracellular enzyme D-AAT and GluRA which participate in production of biodegradable Polly gamma-glutamic acid directly, and AlaRA activity, After collecting biomasses and crushing a biomass by an ultrasonic crusher next it inoculated this invention strain into 5-ml LB liquid medium and 10 hours cultivated at 37 **, it centrifuges and crude enzyme liquid is obtained. An activity fixed quantity of D-AAT makes the crude enzyme liquid obtained by the above react it to D-alanine and alpha-keto glutamic acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and by an enzyme reaction. The activity can be quantified by measuring the quantity of the pyruvic acid which is a reaction product produced, GluRA can analyze the L-glutamic acid produced by the post-enzyme reaction which made crude enzyme liquid react to D-glutamic acid,

alpha-glutamine acid, and PLP into 50mM tris buffer solution (Tris-HCl, pH 8.5) in an optical activity HPLC column. and can make the activity a fixed quantity. AlaRA measures with an absorbance the pyruvic acid produced with alanine dehydrogenase by making D-alanine into a disposition at 340 nm, and makes the activity a fixed quantity.

[0033]Hereafter, this invention is explained more to details through working example. It is for these working example only explaining this invention more concretely, and the range of this invention is not limited by these working example according to the gist of this invention.

[Translation done.]

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EXAMPLE

[Example](Working example 1) ****** which is the tradition beans fermented food produced by the traditional beans bacterial coupling through the decomposition of a microorganism and the separation straw of an identification 1. microorganism which produce Polly gamma-glutamic acid came to hand all over the country, and it was used as a sample. After following the endospore formation-ized process which is heat-treated for 20 minutes with a 60 ** constant temperature bath, and a bacillus has next it added each sample to sterilization distilled water in small quantities and was suspended, The smear of the suspension is carried out to GS plate agar (1.5% L-glutamic acid, 5.0% sugar, 0.27%KH_2PO_4, 0.42%Na_2HPO_4, 0.05%NaCl, and 0.05%$MgSO_4$ and 0.05% BIOKEN) which contains agar 2%, It cultivated for three days to a 37 ** incubator. The biomass which forms the mucosity bacillus colony shown by Polly gamma-glutamic-acid production was separated after culture.
[0035]A possibility that a separation bacillus can be co-cultured by mucosity Polymer Division Polly gamma-glutamic-acid production is taken into consideration, After carrying out the smear according to a continuation thin method on LB culture medium which is a general culture medium which does not produce a polymer. biomass growth carried out pure isolation only of the most flourishing bacillus, and selected with the Polly gamma-glutamic-acid production strain, and biochemical characterization was examined closely using that said strain is morphological and a general culture medium without polymer production of mucosity.
[0036]In order to investigate the constitutive enzyme activity of the enzyme complex which participates in polymer production of the Polly gamma-glutamic-acid production strain obtained by this invention, the separated strain was inoculated into 5-ml LB liquid medium, and 10 hours cultivated it. Said culture medium was centrifuged, biomasses were collected, and after crushing the biomass by the ultrasonic crusher and obtaining crude enzyme liquid, this was used for D-AAT and GluRA which participate in Polly gamma-glutamic-acid production, and AlaRA enzyme activity measurement.
[0037]2., and morphological and it is biochemical characterization (1)   [a microorganism]   Although edible [which was separated in growth and the gestalt characteristic above-mentioned stage of   microorganism], water solubility, biodegradability, and the activity strain from ** ionicity Polly gamma-glutamic-acid Takao formed the colony of the bacillus which takes out high mucosity with GS agar plate background which is a Polly gamma-glutamic-acid production culture medium, By LB agar plate culture medium which does not produce mucosity pile composition, the milky bacillus colony was formed and many of biomasses showed the cylindrical gestalt. Under the temperature influence which it has on biomass growth, growth was nursed in [not less than 30 **] 55 ** or less, and biomass growth was not able to be checked above 60 **.
[0038]When microscope observation was carried out by the exponential phase using LB liquid medium of biomass growth, it is a bacillus of a comparison strain. It was similar with Subtilis (graphic display abbreviation). It was a Gram positive and the sizes of the cell were 0.7 to 0.8X2.0-3.0 micrometers of outlines. RICHINIPOMISU which is other comparison strains expressed the cylindrical pattern that the biomass outside of an exponential phase was thin on the other hand again, and the separation strain of this invention expressed the different biomass outside.
[0039](2) The strain and bacillus by sodium chloride tolerance this invention After 24 hours cultivated the Subtilis fermented-soybeans (B. subtilis natto) strain by LB culture medium by which NaCl of each concentration was added, the absorbance was measured at 660 nm and the growth grade of each strain was measured (drawing 2). From the density range (12%) where the strain by this invention can grow so that it may see by a diagram to a bacillus Compared with the Subtilis fermented soybeans, it turns out that it is the tolerance over a twice [about] as many survival rate, i.e., sodium chloride, as this.
[0040](3) The strain by plasmid content characteristic this invention, bacillus Bacillus Subtilis 168 and the bacillus which are the type strains of Subtilis The plasmid was separated from Subtilis fermented-soybeans IFO3336, and the content propriety was checked (drawing 3). At drawing 3, M is 1kb rudder marker and A is a

bacillus. Subtilis 168, the strain according [B] to this invention, and C express bacillus Subtilis fermented-soybeans IFO3336.

[0041]Although the strain by this invention is a strain which produces Polly gamma-glutamic acid as it sees by a diagram, it turns out that a plasmid like fermented-soybeans IFO3336 is not contained. And the same feature as bacillus Subtilis 168 which is a strain which cannot produce Polly gamma-glutamic acid is seen.

[0042]The strain by this invention can be used also for the host who suited the high manifestation system (secretory production) of the recombination protein which did not contain a plasmid, therefore carried out gene manipulation like [at the time of explaining in full detail].

[0043](4) The spore was dyed and observed, after inoculating the strain by this invention into LB culture medium by which $CoSO_4$ of sporulation characteristic 2mM was added and cultivating for four days at 37 ** (graphic display abbreviation). Bacillus which is the Polly gamma-glutamic-acid production strain with same strain by this invention It has checked that sporogenous ability power had come out notably compared with the Subtilis fermented soybeans.

[0044](5) In addition, the biochemical characterization of the strain by this invention, etc. were investigated using biochemistry characteristic API50CHB and an API20E kit.

[0045]The strains by this invention are gram positive bacteria, do not have the reducing power of a nitrate and do not produce Indore. Gelatin and starch are decomposed, beta **GURIKOSHIDAZE and **-galactosidase are produced, and oxidase is produced. An urease can be produced and it can grow up altogether on golden opportunity base conditions. It expressed as what can use glycerol, galactose, glucose, a shook sirloin, malt sugar, and starch.

[0046]It is as [detailed / it having been morphological and having expressed biochemical characterization to Table 1] the microorganism sorted out by this invention.

[0047]

[Table 1]

特性	本菌株の諸果
グラム染色	陽性
形態	桿状
胞子形成	少し膨張（よく観察すれば）
内生胞子の寸法	0.7～0.8 x 2.0～3.0μm
成長温度	30～55℃
pH6．7での成長	陽性
NaCL10%での成長	陽性
好嫌気条件での成長	陽性
嫌気的条件での成長	陽性
運動性試験	陽性
硝酸塩還元	陰性
インドール形成	陰性
ゼラチン分解	陽性
カタラーゼ形成	陽性
ウレアーゼ生成	陽性
ガラクトシダーゼ生成	陽性

[0048](6) In order to identify more correctly the separation strain obtained by base sequence analysis this invention, gene base sequence analysis of 16S rDNA was carried out.

[0049]First, after amplifying 16S rDNA gene in PCR using N-end primer (5'-AGAGTTTGATCCTGGCTCAG-3') and C-end primer (5'-AGAAAGGAGGTGATCCAGCC-3'), Cloning was carried out to plasmid pT7Blue, and the whole base sequence was determined. It is a bacillus as a result of comparing much 16SrDNA base sequences and homology of a microorganism to whom 16S rDNA base sequence of the microorganism sorted out is reported conventionally. Homology was expressed as Subtilis 99.0% and it has judged as what is located in a system which was illustrated by drawing 4.

[0050](7) The separation strain of this invention shows the characteristic which does not contain a plasmid unlike the usual genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production in spite of identification of the separated strain, however homology high as mentioned above. Such the characteristic shows that the strain by this invention can use gene manipulation for the host who suited the high manifestation system of the recombination protein which led. Unlike a genus Bacillus stock, nitrate reduction power is netative, sporulation is not performed easily but the separation strain of this invention has the characteristic which is not easily derived to manganese ion.

[0051]It is a bacillus natto fermented soybeans and bean paste hot pot (Bacillus.) about the strain of this invention about the characteristic (following working example can explain) of the Polly gamma-glutamic acid which the characteristic and this strain of the above strain itself produce. It classifies into the new strain belonging to Subtilis, and is a bacillus. Subtilis It is named subtilis var.chungkookjang. The name of said strain was made into Bacillus BS-4' (Bacillus sp.BS-4) for convenience, it ****ed to the Biotechnology Division research institute gene bank (KCTC, South Korean Taejon Metropolitan *********** 52 whereabouts) on November 18, 1999, and deposition number KCTC0697BP was given.

[0052](Working example 2) After it inoculated the separation strain of generation this invention of Polly gamma-glutamic acid into the Polly gamma-glutamic-acid production culture medium and 72 hours cultivated at 37 **, the Polly gamma-glutamic-acid content sample solution was acquired by adjusting so that the 2N solution of hydrochloric acid may be added and pH may be set to 3.0. 10 hours made said sample solution settle at 4 **, and polysaccharide in fermented mash was removed, it added so that it might become fermented mash twice the volume of said there about ethanol, and it fully mixed. After 10 hours made mixed liquor settle at 4 **, it centrifuged and the Polly gamma-glutamic-acid sediment was obtained. Add distilled water to said sediment and it was made to dissolve in it, protease was added so that it might be set to 100 ug(s)/ml, and 37 ** humidistat was made to decompose the quality of extracellular protein of 6 hours which carries out a between settlement reaction and exists in a Polly gamma-glutamic-acid sample. It condensed, after removing the glutamic acid which dialyzed and separated this with sufficient quantity of distilled water, and pure Polly gamma-glutamic acid was obtained. The these-refined Polly gamma-glutamic acid measured the presentation and the quantity of production of D and L glutamic acid which were obtained by performing oxidized water decomposition.

[0053]As the productivity of the Polly gamma-glutamic acid which the strain of this invention and the strain used for comparison produce was expressed to Table 2, as for the separation strain of this invention, the liquid medium showed the productivity of 16 g/L. Bacillus separated from fermented soybeans Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a showed the Polly gamma-glutamic-acid productivity of 10 g/L and 9 g/L respectively. In order to compare the productivity of Polly gamma-glutamic acid in a solid medium. After inoculating the bacillus into the plate agar which is a Polly gamma-glutamic-acid production culture medium which contains agar 2% and cultivating for three days at 37 **, Polly gamma-glutamic acid was refined identically to the above-mentioned refining method, and the difference of the productivity of this invention separation strain and a comparison strain was investigated. As for the test result and the separation strain of this invention, 8 mg / plate agar, and RICHIEPOMISU ATCC9945a expressed the productivity of 6 mg / plate agar, and 12 mg / plate agar, and bacillus Subtilis fermented-soybeans IFO3336a checked that this invention separation strain had twice [about] as many productivity as this compared with a comparison strain. The result of having measured the quantity of the Polly gamma-glutamic acid respectively produced per 0.3-mg strain so that it might see with the gel photograph of drawing 5. Bacillus which is a Polly gamma-glutamic-acid production strain of existing [the separation strain of this invention] It can check producing Polly gamma-glutamic acid of very much quantity from Subtilis fermented-soybeans IFO3336a.

[0054]

[Table 2]

[illegible]	[illegible]	[illegible]
[illegible]	16	[illegible]
[illegible]	10	[illegible]
[illegible]	9	[illegible]

[0055](Working example 3) The quantity of production of the Polly gamma-glutamic acid which D of Polly gamma-glutamic acid and the separation strain of stereospecificity investigation this invention of L-glutamic acid produce, and the presentation of D which is a constituent of Polly gamma-glutamic acid, and L-glutamic acid were investigated.

[0056]In order to investigate the percentage of D which is a monomer of Polly gamma-glutamic acid of the amount of Polymer Division which the separation strain of this invention produces, and L-glutamic acid, After making the pure Polly gamma-glutamic-acid sample which 72 hours cultivates with 150 rpm and 37 ** humidistat using the Erlenmeyer flask which is 500 ml which GS production culture medium contained, and could be refined in the above-mentioned refining method and the similar way add and deaerate 6N chloride, 10 hours hydrolyzed at 105 **.

[0057]The amino acid analysis of the above-mentioned hydrolysate uses the concentration gradient using 50mM phosphoric acid buffer solution (pH 7.0) which contains methanol 5%, and methanol. The HPLC column (RexchromeS5-100-ODS, Regis Chem. 4.6mmX25cmX5m. U.S.) analyzed. After separation of the stereoisomeric form made D and the amino-terminus part of L-glutamic acid derivatize using o-phthalaldehyde. In 452 nm (Em)

and 342 nm (Ex). D and L-glutamic acid which are the constituents of Polly gamma-glutamic acid were made a fixed quantity according to the standard curve of D and L-glutamic acid with the fluorescence detector.
[0058]As the result of having investigated the content of D which is a monomer which constitutes the produced Polly gamma-glutamic acid, and L-glutamic acid was expressed to Table 2. The ratios of D/L-glutamic acid from the Polly gamma-glutamic acid which this invention separation strain produces are about 40/60, Bacillus which is a comparison strain In Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a, the ratio of D/L-glutamic acid is 50/50, and the separation bacillus was able to see different monomer percentage.
[0059](Fixed quantity of enzyme activity which participates in Polly gamma-glutamic-acid production)
In order to measure the enzyme activity which participates in Polly gamma-glutamic-acid production of this invention separation strain, It centrifuged, after cultivating a biomass with 37 ** humidistat using LB liquid medium which does not produce a mucosity polymerization agent, and after adjusting crude enzyme liquid with the method which mentioned this above next, the enzyme activity included in crude enzyme liquid was measured.

[0060]It makes the activity of D-AAT react crude enzyme liquid to D-alanine and **-ketoglutaric acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and it by an enzyme reaction. It quantifies by measuring the quantity of the pyruvic acid which is the produced reaction product (Berntsson S, Anal.Chem., 27:1659-1660-1995), The activity of GluRA analyzed and quantified the L-glutamic acid produced by the enzyme reaction in the optical activity HPLC column, after making crude enzyme liquid react to D-glutamic acid, **-ketoglutaric acid, and PLP in 50mM tris buffer solution (Tris-HCl, pH 8.5). The spectrometry of the pyruvic acid which made alanine dehydrogenase react to the L-alanine produced considering D-alanine as a substrate, and was generated was carried out, and alanine racemase activity measurement (Biochemistry, 25:3261-3267,1986) quantified it. Protein content was measured by the Bradford method (Bradford, M., Anal.Biochem., 72:248-254-1976).
[0061]The activity measurement result of the quantity of Polly gamma-glutamic acid, a molecular weight and D, an L-glutamic acid ratio, and an enzyme (D-AAT, GluRA, AlaRA) by which the product from happiness in the next life is carried out of having cultivated the separation strain of this invention with the Erlenmeyer flask was shown in Table 2 and Table 3. Bacillus known as a Polly gamma-glutamic-acid production strain separated from Japanese fermented soybeans in order to compare the characteristic of the Polly gamma-glutamic acid which this invention separation strain produces Subtilis, and the Polly gamma-glutamic-acid quantity of production and enzyme activity of RICHIEPOMISU were measured and displayed.
[0062]
[Table 3]

**	酵素活性 (units/mg タンパク質)		
	D-AAT	GluRA	AlaRA
本発明の菌株	0.809	0.0105	0.189
[illegible] IFO3336a	0.184	0.0840	0.183
[illegible] ATCC9945a	0.197	0.0021	0.000

[0063]As a result of comparing and examining the above enzyme activity, the separation strain of this invention AlaRA, D-Ala and D-Glu of cell growth and Polly gamma-glutamic acid required for production are compounded using GluRA activity, Bacillus It can expect using the activity of D-AAT higher about 3 times than the Subtilis fermented soybeans and RICHIEPOMISU as a thing with the course which compounds D-Glu in large quantities and uses it for production of Polly gamma-glutamic acid directly from D-Ala. Bacillus The Subtilis fermented soybeans and RICHIEPOMISU so that Tables 2 and 3 and drawing 1 may see, It can expect as a thing with the course which compounds glutamic acid required for composition of cell growth and Polly gamma-glutamic acid using high GluRA activity, The separation strain of this invention is a bacillus. It was considered Subtilis fermented-soybeans IFO3336a and RICHIEPOMISU ATCC9945a as a thing with each-other different **** amino-acid-synthesis course (drawing 6). Are drawing 6 and the glutamine:2-oxo guru TAREDO amino mutase and 2 1 A glutamine synthetase. 3 — L-glutamic acid: -- as for the pyruvic acid amino mutase and 4, the D-amino acid amino mutase and 6 are Polly gamma-glutamate synthesis enzymes alanine racemase and 5, and TCA expresses a tricarboxylic acid cycle.
[0064]Namely, bacillus While D-glutamic acid which can be used for composition of Polly gamma-glutamic acid is converted into intracellular in the case of the Subtilis Bacillus natto stock, L-glutamic acid is converted into D-glutamic acid by operation of glutamic-acid racemase and it is made, In the separation strain of this invention, D-glutamic acid is produced from L-glutamic acid by operation of alanine racemase and the D-amino acid amino mutase.
[0065](Working example 4) bacillus which are a separation strain of determination-of-molecular-weight this

invention by comparison (1) electrical-and-electric-equipment **** of the molecular weight of Polly gamma-glutamic acid, and the type strain in a bacillus Subtilis 168 -- and,Bacillus which is a comparison strain In order to measure the molecular weight of the Polly gamma-glutamic acid which Subtilis fermented-soybeans IFO3336 produces, concentration gradient SDS-PAGE was carried out.

[0066]After refining the Polly gamma-glutamic acid produced from each biomass with the refining method explained in full detail in said working example 2, the about 200 ug(s)/ml solution was prepared. After mixing each Polly gamma-glutamic-acid solution 80ul with 5X buffer solution 20ul by which dyeing medicine was added, electric **** was performed by 5 to 20% of concentration gradient polyacrylamide gel. Standard protein and Polly gamma-glutamic acid were dyed for the electric **** completion back of each by a KOMASHI dyeing reagent and methylene blue (drawing 5). At drawing 5, M is standard protein and 1 is a bacillus. The strain according [according to / in Subtilis 168 and 2 / bacillus Subtilis fermented-soybeans IFO3336 / 3] to this invention was expressed.

[0067]Like drawing 5, the separation strain of this invention is a bacillus. It was able to check producing Polly gamma-glutamic acid of a far larger molecular weight than the molecular weight (about 1,000 KDa(s) - 2,000KDa) of the Polly gamma-glutamic acid which the Subtilis fermented soybeans produce.

[0068](2) After cultivating the separation strain of determination-of-molecular-weight this invention by a gel filtration chromatograph (GPC) for five days by GS solid medium, Polly gamma-glutamic acid was refined by the aforementioned method, and the molecular weight was analyzed using the gel penetration chromatograph (Asahipak GS-620 H+Tosoh TSK gel).

[0069]a gel filtration chromatograph -- 50mM salt: -- to the solvent, the rate of flow of the solvent carried out the acetonitrile (4:1) solution with 25 ** column oven at 0.7 ml/m. In the standard substance, polyethylene oxide was used and the molecular weight of Polly gamma-glutamic acid was measured using the refraction index measuring instrument.

[0070]The chromatograph of the test result was illustrated to drawing 7. As a result of analyzing this, as for the Polly gamma-glutamic acid which the separation strain by this invention produces, it turns out that Mw (an average molecular weight, weightaverage molecular weight) is [about 13 million and a molecular-weight-distribution figure (polydispersity)] about 8.0.

[0071]This proves that not only the chisel with a very large molecular weight compared with the thing which other strains produce but its molecular weight distribution of the Polly gamma-glutamic acid which the strain by this invention produces is uniform. Therefore, the Polly gamma-glutamic acid produced from the strain of this invention can be utilized very useful as an object for hydration gel manufacture.

[0072](Working example 5) Polly gamma-glutamic-acid molecular weight change of the strain by this invention which utilized Polly gamma-glutamic-acid decomposition activity measurement GPC of the strain by this invention, and followed culture time progress was investigated.

[0073]Cultivating the separation strain of this invention by GS solid medium, Polly gamma-glutamic acid was respectively refined by the aforementioned method on 1, 3, and the 5th at the time of progress, and a molecular weight and molecular weight distribution were investigated using GPC (Table 4).

[0074]

[Table 4]

培養時間 (日)	平均分子量	分子量分布
1	1．204×10⁴	7．9
3	1．197×10⁴	7．8
5	1．311×10⁴	8．0

[0075]It turns out that the Polly gamma-glutamic acid compounded by the strain by this invention hardly changes an average molecular weight and molecular weight distribution even if culture time passes so that it may see in Table 4. therefore, strain bacillus by this invention Subtilis or [that a natto fermented soybeans and bean paste hot pot does not have Polly gamma-glutamic-acid decomposition activity] -- or it can be judged that there is not almost it.

[Translation done.]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1]It is a figure showing the cell wall by various intracellular enzymes, and the constituent synthetic pathway of Polly gamma-glutamic acid.
[Drawing 2]Bacillus of this invention Subtilis A natto fermented soybeans and bean paste hot pot and bacillus Subtilis It is a graph which compares the sodium chloride tolerance of fermented soybeans.
[Drawing 3]Bacillus of this invention Subtilis It is a gel electrical-and-electric-equipment **** photograph which shows the plasmid existence propriety of a natto fermented soybeans and bean paste hot pot and other comparison strains. M is 1kb rudder marker and A is a bacillus. As for Subtilis 168 and B, the strain by this invention and C are bacilli. Subtilis fermented-soybeans IFO3336 is expressed.
[Drawing 4]Bacillus of this invention strain based on 16S rDNA base sequence Subtilis It is a distribution diagram of a natto fermented soybeans and bean paste hot pot.
[Drawing 5]Bacillus of this invention Subtilis It is a concentration gradient SDS-PAGE gel electrical-and-electric-equipment **** photograph of the Polly gamma-glutamic acid produced by the natto fermented soybeans and bean paste hot pot and other comparison strains. M is a standard protein marker and 1 is a bacillus. Subtilis 168 and 2 is a bacillus. Subtilis fermented-soybeans IFO3336 and 3 expressed the strain by this invention.
[Drawing 6]Bacillus of this invention Subtilis It is the figure which expressed ly the Polly gamma-glutamic-acid biosynthetic path of the natto fermented soybeans and bean paste hot pot. 1 -- glutamine: -- the 2-oxo guru TAREDO amino mutase and 2 -- a glutamine synthetase. 3 -- L-glutamic acid: -- as for the pyruvic acid amino mutase and 4, the D-amino acid amino mutase and 6 are Polly gamma-glutamate synthesis enzymes alanine racemase and 5, and TCA expresses a tricarboxylic acid cycle.
[Drawing 7]bacillus of this invention Subtilis the gel chromatograph result of the Polly gamma-glutamic acid which the natto fermented soybeans and bean paste hot pot produced -- a table -- the bottom is a graph.
[Layout Table]
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E01-009
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KR2001-1481
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Kopatent In 1.71
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DNA
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Artificial Sequence
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Single stranded oligonucleotide primer
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2
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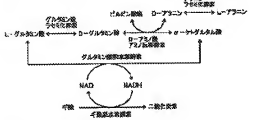
tgatccagcc

[Translation done.]

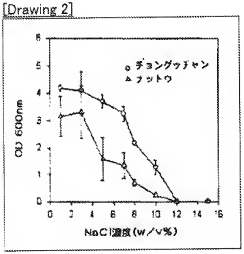
* NOTICES *

DRAWINGS

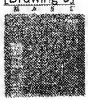
[Drawing 1]



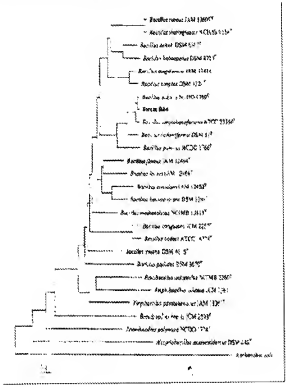
[Drawing 2]



[Drawing 3]

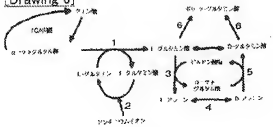


[Drawing 4]

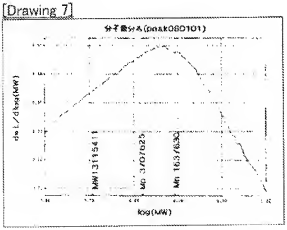


[Drawing 5]

[Drawing 6]



[Drawing 7]



[Translation done.]

＊NOTICES＊

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

WRITTEN AMENDMENT

[Written Amendment]
[Filing date]Heisei 14(2002) February 8 (2002.2.8)
[Amendment 2]
[Document to be Amended]Description
[Item(s) to be Amended]DETAILED DESCRIPTION
[Method of Amendment]Change
[Proposed Amendment]
[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention straw. The salt-tolerant bacillus Subtilis (bacillus subtlis) natto fermented soybeans and bean paste hot pot stock separated from the natto fermented soybeans and bean paste hot pot (******) which is a tradition beans fermented food of used South Korea (KCTC Bacillus subtilis var.chungkookjang) It is related with Polly gamma-glutamic acid which is an extracellular secretion nature polymer produced from 0697BP and said strain, and is edible, water solubility, negative ion nature, and a biodegradable polymer substance. The D-amino acid transaminase which is an enzyme in which this invention makes keto acid transfer the amino group of D-amino acid to details more (D-amino acid aminotransferase:EC2.6.1.21). (It is hereafter called D-AAT for short), The nature object formation of the opposite sex of an alanine and glutamic acid. Glutamic-acid racemase (Glutamate racemase:EC5.1.1.3; call for short the following GluRA) and alanine racemase (Alanine racemase; call for short the following AlaRA) which are enzymes which carry out a catalyst. And it is related with the Polly gamma-glutamic acid produced by the new strain which produces Polly gamma-glutamic acid out of a cell with intracellular enzyme complexes, such as a Polly gamma-glutamate synthesis enzyme (Poly-gamma-glutamate synthetase), and said strain. As illustrated to drawing 1, many enzymes are participating in composition of Polly gamma-glutamic acid.
[0002]
[Description of the Prior Art]Poliy gamma-glutamic acid is the polymer which carried out Polly gamma-glutamyl (gamma-glutamiyl) combination, and D and L-glutamic acid as mucous material. It is produced from the genus Bacillus stock separated from "KINEMA" etc. which are the "natto fermented soybeans and bean paste hot pot" (******) which is a tradition beans fermented food of South Korea using straw, the "fermented soybeans" which are Japanese tradition beans fermented foods, and a tradition beans fermented food of Nepal. The Polly gamma-glutamic acid produced from said genus Bacillus stock Edible, It can use for the raw material substance for the natural decomposition nature plastic manufacture by the desiccant, the moisturizer and the raw material of cosmetics, and the affinity of an ester derivative by water solubility, negative ion nature, and a biodegradable polymer substance (molecular weight: 100,000-2,000,000).
[0003]Recently, the research which got interested in production of Polly gamma-glutamic acid, development and the soluble fiber of a heat-resistant plastic, film production according to the substitutive-goods raw material of a difficulty degradable polymer and an esterification reaction about use, etc. has been advancing actively mainly by industrialized nations. The development of hydro-gel (hydrogel) and industrialization research by the change-in-physical-properties research and the crosslinking bond agent which are caused in Polly gamma-glutamic acid at the time of gamma irradiation are promoted. The influence of manganese ion which it will have on the presentation of Polly gamma-glutamic acid, and Polly gamma-glutamic-acid production if an example is given, The research to use to a water-soluble polymer and research (Biosci.Biotechnol.Biochem., 60(8):1239-1242-1996) on development of the low-water-flow solubility plastic by composition of an ester derivative, and bacillus by ultrasonic decomposition. The practical use (JP,H6-32742,A) to health food with the ********** curative effect as the Polly gamma-glutamic-acid production by Subtilis and a calcium resolvent, etc. see.

[0004]In addition, the effect (Euro patent No. 838160) of decreasing the phosphorus content of a drainage system and decreasing water pollution, Biodegradable adsorbent resin with the high gelation properties by radiation irradiation and absorptivity is manufactured, and there is a report of application (JP,H10-251402,A), practical use (JP,H7-300522,A, JP,H6-322358,A), etc. to sanitary goods, foodstuffs, and horticulture industry of a diaper etc. the use (JP,H7-138364,A.) as the solidification biodegradable fiber by the dissolution of Polly gamma-glutamic acid, precipitate, and desiccation, a film, and a film formation agent There is also a report to JP,H5-117388,A, polymer for drug carriers (JP,H6-92870,A, JP,H6-256220,A), etc.

[0005]On the other hand, in South Korea with fundamental researches, such as efficient production (South Korean patent application No. 3404 [1997 to], South Korean patent application No. 67605 [1997 to]), a characteristic improvement, etc. of Polly gamma-glutamic acid. The application study which is going to use for the source material of cosmetics the Polly gamma-glutamic acid which a bacillus Bacillus natto stock produces by the Pacific Ocean, Inc. occurs.

[0006]

[Problem(s) to be Solved by the Invention]However, the molecular weights of the Polly gamma **GURUTAMIN acid obtained by the method using the conventional genus Bacillus stock are 100,000-2,000,000, and for a desiccant, a moisturizer, or natural decomposition nature plastic manufacture, The method that productivity was higher was called for the direction which produces the Poliy gamma **GURUTAMIN acid of Polymer Division more.

[0007]Therefore, this invention aims to let a molecular weight provide the method of producing more 2,000,000 or more Polly gamma **GURUTAMIN acid to a large quantity using a genus Bacillus stock.

[0008]

[Means for Solving the Problem]Salt-tolerant strain bacillus separated from a natto fermented soybeans and bean paste hot pot (******) of a South Korean tradition beans fermented food as a result of this invention persons' inquiring wholeheartedly to achieve the above objects Subtilis A natto fermented soybeans and bean paste hot pot stock, it finds out producing Polly gamma-glutamic acid of the amount of Polymer Division at high concentration, and came to complete this invention based on these knowledge.

[0009]Namely, this invention Polly gamma-glutamic acid which is a biodegradable polymer substance is produced, and it is salt tolerance, Bacillus which sporulation was difficult, and did not contain a plasmid in the strain itself, but was separated from a natto fermented soybeans and bean paste hot pot (******) Subtilis It is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).

[0010]Bacillus whose nitrate reduction power the above-mentioned strain is netative in the above-mentioned invention and whose deposition number is KCTC0697BP Subtilis It is preferred that it is a natto fermented soybeans and bean paste hot pot stock (Bacillus subtilis var.chungkookjang).

[0011]Another of an invention is a recombination protein production method using the above-mentioned strain as a host.

[0012]Furthermore, another of an invention is a manufacturing method of Polly gamma-glutamic acid using the above-mentioned strain.

[0013]In the above-mentioned invention, it is preferred to include the following stage.

(a) A stage which cultivates the above-mentioned strain and acquires Polly gamma-glutamic acid

(b) A stage which acquires a Polly gamma-glutamic-acid sediment next it removed and carried out solvent extraction of the polysaccharide and centrifuged it with Polly gamma-glutamic-acid content liquid which acquired [above-mentioned]

(c) A stage into which processed to protease and extracellular nature protein was made to disassemble after dissolving the above-mentioned Polly gamma-glutamic-acid precipitate

(d) A stage condensed after dialyzing and removing isolation glutamic acid

[0014]According to this invention, how a molecular weight produces efficiently 2,000,000 or more Polly gamma **GURUTAMIN acid can be provided.

[0015]Furthermore, another of an invention is Polly gamma-glutamic acid which is manufactured by the above-mentioned strain and characterized by a molecular weight being 2,000 or more kDa.

[0016]According to this invention, the Polly gamma **GURUTAMIN acid which fitted a desiccant, a moisturizer, and natural decomposition nature plastic manufacture rather than the Polly gamma **GURUTAMIN acid produced by the conventional genus Bacillus stock can be provided.

[0017]Furthermore, another of an invention is the cosmetics containing the above-mentioned Polly gamma-glutamic acid.

[0018]Furthermore, another of an invention is the health food containing the above-mentioned Polly gamma-glutamic acid.

[0019]Furthermore, another of an invention is a drink containing the above-mentioned Polly gamma-glutamic

acid.
[0020]Furthermore, another of an invention is the drugs containing the above-mentioned Polly gamma-glutamic acid.
[0021]According to this invention, cosmetics, health food, a drink, drugs, etc. which contain Polly gamma-glutamic acid of the amount of Polymer Division conventionally can be provided.
[0022]
[Embodiment of the Invention]Hereafter, this invention is explained more concretely.
[0023](Separation and identification of a strain) Bacillus which is a new strain of this invention which produced Polly gamma-glutamic acid of edible, water solubility, negative ion nature, and biodegradability with high yield, and had salt tolerance Subtilis Separation of a natto fermented soybeans and bean paste hot pot and the method of identification are as follows.
[0024]It is produced in the Republic of Korea, and in order to separate a strain with a Polly gamma-glutamic-acid high throughput from the sample of 20 kinds of ****** which are a tradition beans fermented food using straw, various ****** samples are heat-treated for 20 minutes with a 60 ** constant temperature bath, after being suspended to distilled water, the colony pure isolation of the bacillus which cultivates for three days with 37 ** humidistat, and expresses high viscosity after carrying out the smear of said suspension small quantity made to heat-treat to the Polly gamma-glutamic-acid production agar plate culture medium (GS) containing 1.5% of L-glutamic acid -- it carries out. After carrying out subculture twice for these separation bacillus repeatedly using the same above double grounds, a strain with the most active biomass growth is separated in the bacillus colony from which high viscosity is taken out by production of Polly gamma-glutamic acid. Although said separated Polly gamma-glutamic-acid high throughput strain forms a milky bacillus colony from LB plate agar which contains agar 2%, this is cultivated at 37 ** by a **** thin method for 20 hours, and the strain it becomes active [growth of a biomass] most [strain] is separated.
[0025]this invention strain separated by the above-mentioned method is morphological, and the physiological character is as follows.
[0026]When cultivating by LB agar plate culture medium, opalescence carries out bacillus colony formation of this invention strain, and it has the characteristic that biomass growth becomes slow in the culture temperature of not less than 55 ** as the gram positive bacteria with active growth of a biomass on not less than 37 ** golden opportunity conditions. Bacillus with the common this invention strain It is a salt-tolerant strain producible also by 9.0% of salt (NaCl) concentration higher than the salt tolerance concentration which Subtilis has, the result of having made the comparative analysis of the 16S rDNA base rank of this invention separation strain to the strain 16S rDNA base sequence in a bacillus conventionally -- the homology (99.0%) of bacillus Subtilis (Bacillus subtilis) and very high 16S rDNA base sequence -- a table -- the bottom.
[0027]However, in spite of the above high homology, this invention -- bacillus in a new separation bacillus Subtilis . A natto fermented soybeans and bean paste hot pot can be used also for the strain which suited the high manifestation system of the recombination protein which did not contain the plasmid unlike the **** genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production, and let gene manipulation pass. The separation strain by this invention is a safe microorganism in which edible is possible. Therefore, for example, a vaccine can be made to be able to reveal by the ability to make said strain into a host (making the antigen portion of for example, a pig diarrhea virus reveal), and the strain itself can be used for the feed additives for a diarrhea disease therapy or prevention.
[0028]That is, oral vaccine development is attained using the strain of this invention.
[0029]Unlike other genus Bacillus stocks, nitrate reduction power is netative, sporulation does not happen easily and the separation strain of this invention has the characteristic which is not easily derived by manganese ion, either. It is a bacillus about the strain of the aforementioned result to this invention. It classifies into Subtilis and is a bacillus. Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) is named. The name of said strain was made into 'Bacillus BS-4' (Bacillus sp.BS-4) for convenience, and it ****ed to the Biotechnology Division research institute gene bank (KCTC, Taejon Metropolitan ********** 52 whereabouts) as an accession number of KCTC0697BP on November 18, 1999.
[0030](Analysis of Polly gamma-glutamic acid and activity measurement of an intervention enzyme) A fixed quantity of the Polly gamma-glutamic acid produced by said strain, D of a polymer, and the check of an L-glutamic acid presentation are carried out as follows.
[0031]Bacillus Subtilis After cultivating a natto fermented soybeans and bean paste hot pot, liquid, such as an upper group which centrifuged the culture medium and Polly gamma-glutamic acid contained, is separated, and D and L-glutamic acid are separated using the crepuscular-rays study activity HPLC column which added high concentration chloride here and was hydrolyzed at the elevated temperature. In order to ask for a standard curve, the refined Polly gamma-glutamic-acid sample was also analyzed by the same method. The content of the

Polly gamma-glutamic acid which calculated the correction value over the isolation L-glutamic acid which carried out semi- [of the substance which passed the column] to D and an L-glutamic acid standard curve, and was added to the initial culture medium quality and after quantifying, and was produced purely is calculated.
[0032]For measurement of intracellular enzyme D-AAT and GluRA which participate in production of biodegradable Polly gamma-glutamic acid directly, and AlaRA activity. After collecting biomasses and crushing a biomass by an ultrasonic crusher next it inoculated this invention strain into 5-ml LB liquid medium and 10 hours cultivated at 37 **, it centrifuges and crude enzyme liquid is obtained. An activity fixed quantity of D-AAT makes the crude enzyme liquid obtained by the above react it to D-alanine and alpha-keto glutamic acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and by an enzyme reaction. The activity can be quantified by measuring the quantity of the pyruvic acid which is a reaction product produced. GluRA can analyze the L-glutamic acid produced by the post-enzyme reaction which made crude enzyme liquid react to D-glutamic acid, alpha-glutamine acid, and PLP into 50mM tris buffer solution (Tris-HCl, pH 8.5) in an optical activity HPLC column, and can make the activity a fixed quantity. AlaRA measures with an absorbance the pyruvic acid produced with alanine dehydrogenase by making D-alanine into a disposition at 340 nm, and makes the activity a fixed quantity.
[0033]Hereafter, this invention is explained more to details through working example. It is for these working example only explaining this invention more concretely, and the range of this invention is not limited by these working example according to the gist of this invention.
[0034]
[Example](Working example 1) Decomposition and identification of a microorganism which produce Polly gamma-glutamic acid
1. Separation of microorganism
****** which is the tradition beans fermented food produced by the traditional beans bacterial coupling through straw came to hand all over the country, and it was used as a sample. After following the endospore formation-ized process which is heat-treated for 20 minutes with a 60 ** constant temperature bath, and a bacillus has next it added each sample to sterilization distilled water in small quantities and was suspended. The smear of the suspension is carried out to GS plate agar (1.5% L-glutamic acid, 5.0% sugar, 0.27%KH_2PO_4, 0.42%Na_2HPO_4, 0.05% NaCl, and 0.05%$MgSO_4$ and 0.05% BIOKEN) which contains agar 2%, It cultivated for three days to a 37 ** incubator. The biomass which forms the mucosity bacillus colony shown by Polly gamma-glutamic-acid production was separated after culture.
[0035]A possibility that a separation bacillus can be co-cultured by mucosity Polymer Division Polly gamma-glutamic-acid production is taken into consideration, After carrying out the smear according to a continuation thin method on LB culture medium which is a general culture medium which does not produce a polymer, biomass growth carried out pure isolation only of the most flourishing bacillus, and selected with the Polly gamma-glutamic-acid production strain, and biochemical characterization was examined closely using that said strain is morphological and a general culture medium without polymer production of mucosity.
[0036]In order to investigate the constitutive enzyme activity of the enzyme complex which participates in polymer production of the Polly gamma-glutamic-acid production strain obtained by this invention, the separated strain was inoculated into 5-ml LB liquid medium, and 10 hours cultivated it. Said culture medium was centrifuged, biomasses were collected, and after crushing the biomass by the ultrasonic crusher and obtaining crude enzyme liquid, this was used for D-AAT and GluRA which participate in Polly gamma-glutamic-acid production, and AlaRA enzyme activity measurement.
[0037]2.. and morphological and it is biochemical characterization. [a microorganism]
(1) Growth and the gestalt characteristic of a microorganism
Although edible [which was separated in the above-mentioned stage], water solubility, biodegradability, and the activity strain from ** ionicity Polly gamma-glutamic-acid Takao formed the colony of the bacillus which takes out high mucosity with GS agar plate background which is a Polly gamma-glutamic-acid production culture medium, By LB agar plate culture medium which does not produce mucosity pile composition, the milky bacillus colony was formed and many of biomasses showed the cylindrical gestalt. Under the temperature influence which it has on biomass growth, growth was nursed in [not less than 30 **] 55 ** or less, and biomass growth was not able to be checked above 60 **.
[0038]When microscope observation was carried out by the exponential phase using LB liquid medium of biomass growth, it is a bacillus of a comparison strain. It was similar with Subtilis (graphic display abbreviation). It was a Gram positive and the sizes of the cell were 0.7 to 0.8X2.0-3.0 micrometers of outlines. RICHINIPOMISU which is other comperison strains expressed the cylindrical pattern that the biomass outside of an exponential phase was thin on the other hand again, and the separation strain of this invention expressed the different biomass

outside.

[0039](2) Sodium chloride tolerance

The strain and bacillus by this invention After 24 hours cultivated the Subtilis fermented-soybeans (B. subtilis natto) strain by LB culture medium by which NaCl of each concentration was added, the absorbance was measured at 660 nm and the growth grade of each strain was measured (drawing 2). From the density range (12%) where the strain by this invention can grow so that it may see by a diagram to a bacillus Compared with the Subtilis fermented soybeans, it turns out that it is the tolerance over a twice [about] as many survival rate, i.e., sodium chloride, as this.

[0040](3) Plasmid content characteristic

The strain by this invention, bacillus Bacillus Subtilis 168 and the bacillus which are the type strains of Subtilis The plasmid was separated from Subtilis fermented-soybeans IFO3336, and the content propriety was checked (drawing 3). At drawing 3, M is 1kb rudder marker and A is a bacillus. Subtilis 168, the strain according [B] to this invention, and C express bacillus Subtilis fermented-soybeans IFO3336.

[0041]Although the strain by this invention is a strain which produces Polly gamma-glutamic acid as it sees by a diagram, it turns out that a plasmid like fermented-soybeans IFO3336 is not contained. And the same feature as bacillus Subtilis 168 which is a strain which cannot produce Polly gamma-glutamic acid is seen.

[0042]The strain by this invention can be used also for the host who suited the high manifestation system (secretory production) of the recombination protein which did not contain a plasmid, therefore carried out gene manipulation like [at the time of explaining in full detail].

[0043](4) Sporulation characteristic

The spore was dyed and observed, after inoculating the strain by this invention into LB culture medium by which $CoSO_4$ of 2mM was added and cultivating for four days at 37 ** (graphic display abbreviation). Bacillus which is the Polly gamma-glutamic-acid production strain with same strain by this invention It has checked that sporogenous ability power had come out notably compared with the Subtilis fermented soybeans.

[0044](5) In addition, the biochemistry characteristic

The biochemical characterization of the strain by this invention, etc. were investigated using API50CHB and an API20E kit.

[0045]The strains by this invention are gram positive bacteria, do not have the reducing power of a nitrate and do not produce Indore. Gelatin and starch are decomposed, beta **GURIKOSHIDAZE and **-galactosidase are produced, and oxidase is produced. An urease can be produced and it can grow up altogether on golden opportunity base conditions. It expressed as what can use glycerol, galactose, glucose, a shook sirloin, malt sugar, and starch.

[0046]It is as [detailed / it having been morphological and having expressed biochemical characterization to Table 1] the microorganism sorted out by this invention.

[0047]

[Table 1]

[illegible]	[illegible]
グラム染色	[illegible]
形態	[illegible]
胞子形成	[illegible]
内性胞子の形態	0.7~0.8 x 2.0~3.0μm
成長温度	30~55℃
pH9.7での成長	[illegible]
NaCl 15%での成長	[illegible]
好気的条件での成長	[illegible]
嫌気的条件での成長	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
インドール形成	[illegible]
オキシダーゼ生成	[illegible]
カタラーゼ生成	[illegible]
ウレアーゼ生成	[illegible]
βガラクトシダーゼ生成	[illegible]

[0048](6) Base sequence analysis

In order to identify more correctly the separation strain obtained by this invention, gene base sequence analysis

of 16S rDNA was carried out.
[0049]First, after amplifying 16S rDNA gene in PCR using N-end primer (5'-AGAGTTTGATCCTGGCTCAG-3') and C-end primer (5'-AGAAAGGAGGTGATCCAGCC-3'), Cloning was carried out to plasmid pT7Blue, and the whole base sequence was determined. It is a bacillus as a result of comparing much 16SrDNA base sequences and homology of a microorganism to whom 16S rDNA base sequence of the microorganism sorted out is reported conventionally. Homology was expressed as Subtilis 99.0% and it has judged as what is located in a system which was illustrated by drawing 4.
[0050](7) Identification of the separated strain
However, the separation strain of this invention shows the characteristic which does not contain a plasmid unlike the usual genus Bacillus stock which can be conventionally used for Polly gamma-glutamic-acid production in spite of homology high as mentioned above. Such the characteristic shows that the strain by this invention can use gene manipulation for the host who suited the high manifestation system of the recombination protein which led. Unlike a genus Bacillus stock, nitrate reduction power is netative, sporulation is not performed easily but the separation strain of this invention has the characteristic which is not easily derived to manganese ion.
[0051]It is a bacillus natto fermented soybeans and bean paste hot pot (Bacillus,) about the strain of this invention about the characteristic (following working example can explain) of the Polly gamma-glutamic acid which the characteristic and this strain of the above strain itself produce. It classifies into the new strain belonging to Subtilis, and is a bacillus. Subtilis It is named subtilis var.chungkookjang. The name of said strain was made into Bacillus BS-4' (Bacillus sp.BS-4) for convenience. it ****ed to the Biotechnology Division research institute gene bank (KCTC, South Korean Taejon Metropolitan ********** 52 whereabouts) on November 18, 1999. and deposition number KCTC0697BP was given.
[0052](Working example 2) Generation of Polly gamma-glutamic acid
After it inoculated the separation strain of this invention into the Polly gamma-glutamic-acid production culture medium and 72 hours cultivated at 37 **, the Polly gamma-glutamic-acid content sample solution was acquired by adjusting so that the 2N solution of hydrochloric acid may be added and pH may be set to 3.0. 10 hours made said sample solution settle at 4 **, and polysaccharide in fermented mash was removed, it added so that it might become fermented mash twice the volume of said there about ethanol, and it fully mixed. After 10 hours made mixed liquor settle at 4 **, it centrifuged and the Polly gamma-glutamic-acid sediment was obtained. Add distilled water to said sediment and it was made to dissolve in it, protease was added so that it might be set to 100 ug(s)/ml, and 37 ** humidistat was made to decompose the quality of extracellular protein of 6 hours which carries out a between settlement reaction and exists in a Polly gamma-glutamic-acid sample. It condensed, after removing the glutamic acid which dialyzed and separated this with sufficient quantity of distilled water, and pure Polly gamma-glutamic acid was obtained. The these-refined Polly gamma-glutamic acid measured the presentation and the quantity of production of D and L glutamic acid which were obtained by performing oxidized water decomposition.
[0053]As the productivity of the Polly gamma-glutamic acid which the strain of this invention and the strain used for comparison produce was expressed to Table 2, as for the separation strain of this invention, the liquid medium showed the productivity of 16 g/L. Bacillus separated from fermented soybeans Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a showed the Polly gamma-glutamic-acid productivity of 10 g/L and 9 g/L respectively. In order to compare the productivity of Polly gamma-glutamic acid in a solid medium, After inoculating the bacillus into the plate agar which is a Polly gamma-glutamic-acid production culture medium which contains agar 2% and cultivating for three days at 37 **, Polly gamma-glutamic acid was refined identically to the above-mentioned refining method, and the difference of the productivity of this invention separation strain and a comparison strain was investigated. As for the test result and the separation strain of this invention, 8 mg / plate agar, and RICHIEPOMISU ATCC9945a expressed the productivity of 6 mg / plate agar, and 12 mg / plate agar, and bacillus Subtilis fermented-soybeans IFO3336a checked that this invention separation strain had twice [about] as many productivity as this compared with a comparison strain. The result of having measured the quantity of the Polly gamma-glutamic acid respectively produced per 0.3-mg strain so that it might see with the gel photograph of drawing 5, Bacillus which is a Polly gamma-glutamic-acid production strain of existing [the separation strain of this invention] It can check producing Polly gamma-glutamic acid of very much quantity from Subtilis fermented-soybeans IFO3336a.
[0054]
[Table 2]

[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

[0055](Working example 3) D of Polly gamma-glutamic acid, stereospecificity investigation of L-glutamic acid The quantity of production of the Polly gamma-glutamic acid which the separation strain of this invention produces, and the presentation of D which is a constituent of Polly gamma-glutamic acid, and L-glutamic acid were investigated.

[0056]In order to investigate the percentage of D which is a monomer of Polly gamma-glutamic acid of the amount of Polymer Division which the separation strain of this invention produces, and L-glutamic acid, After making the pure Polly gamma-glutamic-acid sample which 72 hours cultivates with 150 rpm and 37 ** humidistat using the Erlenmeyer flask which is 500 ml which GS production culture medium contained, and could be refined in the above-mentioned refining method and the similar way add and deaerate 6N chloride, 10 hours hydrolyzed at 105 **.

[0057]The amino acid analysis of the above-mentioned hydrolysate uses the concentration gradient using 50mM phosphoric acid buffer solution (pH 7.0) which contains methanol 5%, and methanol. The HPLC column (RexchromeS5-100-ODS, Regis Chem, 4.6mmX25cmX5m, U.S.) analyzed. After separation of the stereoisomeric form made D and the amino-terminus part of L-glutamic acid derivatize using o-phthalaldehyde, In 452 nm (Em) and 342 nm (Ex), D and L-glutamic acid which are the constituents of Polly gamma-glutamic acid were made a fixed quantity according to the standard curve of D and L-glutamic acid with the fluorescence detector.

[0058]As the result of having investigated the content of D which is a monomer which constitutes the produced Polly gamma-glutamic acid, and L-glutamic acid was expressed to Table 2, The ratios of D/L-glutamic acid from the Polly gamma-glutamic acid which this invention separation strain produces are about 40/60, Bacillus which is a comparison strain In Subtilis fermented-soybeans IFO3336a and RICHIEPOMISUATCC9945a, the ratio of D/L-glutamic acid is 50/50, and the separation bacillus was able to see different monomer percentage.

[0059](Fixed quantity of enzyme activity which participates in Polly gamma-glutamic-acid production) In order to measure the enzyme activity which participates in Polly gamma-glutamic-acid production of this invention separation strain, It centrifuged, after cultivating a biomass with 37 ** humidistat using LB liquid medium which does not produce a mucosity polymerization agent, and after adjusting crude enzyme liquid with the method which mentioned this above next, the enzyme activity included in crude enzyme liquid was measured.

[0060]It makes the activity of D-AAT react crude enzyme liquid to D-alanine and **-ketoglutaric acid into a 0.1M tris buffer solution (Tris-HCl, pH 8.5), and it by an enzyme reaction. It quantifies by measuring the quantity of the pyruvic acid which is the produced reaction product (Berntsson S, Anal.Chem., 27:1659-1660-1995). The activity of GluRA analyzed and quantified the L-glutamic acid produced by the enzyme reaction in the optical activity HPLC column, after making crude enzyme liquid react to D-glutamic acid, **-ketoglutaric acid, and PLP in 50mM tris buffer solution (Tris-HCl, pH 8.5). The spectrometry of the pyruvic acid which made alanine dehydrogenase react to the L-alanine produced considering D-alanine as a substrate, and was generated was carried out, and alanine racemase activity measurement (Biochemistry, 25:3261-3267,1986) quantified it. Protein content was measured by the Bradford method (Bradford, M., Anal.Biochem., 72:248-254-1976).

[0061]The activity measurement result of the quantity of Polly gamma-glutamic acid, a molecular weight and D, an L-glutamic acid ratio, and an enzyme (D-AAT, GluRA, AlaRA) by which the product from happiness in the next life is carried out of having cultivated the separation strain of this invention with the Erlenmeyer flask was shown in Table 2 and Table 3. Bacillus known as a Polly gamma-glutamic-acid production strain separated from Japanese fermented soybeans in order to compare the characteristic of the Polly gamma-glutamic acid which this invention separation strain produces Subtilis, and the Polly gamma-glutamic-acid quantity of production and enzyme activity of RICHIEPOMISU were measured and displayed.

[0062]

[Table 3]

[illegible]	[illegible]		
	D-AAT	GluRA	AlaRA
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

[0063]As a result of comparing and examining the above enzyme activity, the separation strain of this invention AlaRA, D-Ala and D-Glu of cell growth and Polly gamma-glutamic acid required for production are compounded using GluRA activity. Bacillus It can expect using the activity of D-AAT higher about 3 times than the Subtilis fermented soybeans and RICHIEPOMISU as a thing with the course which compounds D-Glu in large quantities and uses it for production of Polly gamma-glutamic acid directly from D-Ala. Bacillus The Subtilis fermented soybeans and RICHIEPOMISU so that Tables 2 and 3 and drawing 1 may see, It can expect as a thing with the course which compounds glutamic acid required for composition of cell growth and Polly gamma-glutamic acid using high GluRA activity. The separation strain of this invention is a bacillus. It was considered Subtilis fermented-soybeans IFO3336a and RICHIEPOMISU ATCC9945a as a thing with each-other different **** amino-acid-synthesis course (drawing 6). Are drawing 6 and the glutamine:2-oxo guru TAREDO amino mutase and 2 1 A glutamine synthetase, 3 -- L-glutamic acid: -- as for the pyruvic acid amino mutase and 4, the D-amino acid amino mutase and 6 are Polly gamma-glutamate synthesis enzymes alanine racemase and 5, and TCA expresses a tricarboxylic acid cycle.

[0064]Namely, bacillus While D-glutamic acid which can be used for composition of Polly gamma-glutamic acid is converted into intracellular in the case of the Subtilis Bacillus natto stock, L-glutamic acid is converted into D-glutamic acid by operation of glutamic-acid racemase and it is made, In the separation strain of this invention, D-glutamic acid is produced from L-glutamic acid by operation of alanine racemase and the D-amino acid amino mutase.

[0065](Working example 4) Comparison of the molecular weight of Polly gamma-glutamic acid

(1) The determination of molecular weight by electric ****

Bacillus which are a separation strain of this invention, and the type strain in a bacillus Subtilis 168 and bacillus which is comparison strains In order to measure the molecular weight of the Polly gamma-glutamic acid which Subtilis fermented-soybeans IFO3336 produces, concentration gradient SDS-PAGE was carried out.

[0066]After refining the Polly gamma-glutamic acid produced from each biomass with the refining method explained in full detail in said working example 2, the about 200 ug(s)/ml solution was prepared. After mixing each Polly gamma-glutamic-acid solution 80ul with 5X buffer solution 20ul by which dyeing medicine was added, electric **** was performed by 5 to 20% of concentration gradient polyacrylamide gel. Standard protein and Polly gamma-glutamic acid were dyed for the electric **** completion back of each by a KOMASHI dyeing reagent and methylene blue (drawing 5). At drawing 5, M is standard protein and 1 is a bacillus. The strain according [according to / in Subtilis 168 and 2 / bacillus Subtilis fermented-soybeans IFO3336 / 3] to this invention was expressed.

[0067]Like drawing 5, the separation strain of this invention is a bacillus. It was able to check producing Polly gamma-glutamic acid of a far larger molecular weight than the molecular weight (about 1,000 KDa(s) - 2,000KDa) of the Polly gamma-glutamic acid which the Subtilis fermented soybeans produce.

[0068](2) After cultivating the separation strain of determination-of-molecular-weight this invention by a gel filtration chromatograph (GPC) for five days by GS solid medium, Polly gamma-glutamic acid was refined by the aforementioned method, and the molecular weight was analyzed using the gel penetration chromatograph (Asahipak GS-620 H+Tosoh TSK gel).

[0069]a gel filtration chromatograph -- 50mM salt: -- to the solvent, the rate of flow of the solvent carried out the acetonitrile (4:1) solution with 25 ** column oven at 0.7 ml/m. In the standard substance, polyethylene oxide was used and the molecular weight of Polly gamma-glutamic acid was measured using the refraction index measuring instrument.

[0070]The chromatograph of the test result was illustrated to drawing 7. As a result of analyzing this, as for the Polly gamma-glutamic acid which the separation strain by this invention produces, it turns out that Mw (an average molecular weight, weightaverage molecular weight) is [about 13 million and a molecular-weight-distribution figure (polydispersity)] about 8.0.

[0071]This proves that not only the chisel with a very large molecular weight compared with the thing which other strains produce but its molecular weight distribution of the Polly gamma-glutamic acid which the strain by this invention produces is uniform. Therefore, the Polly gamma-glutamic acid produced from the strain of this invention can be utilized very useful as an object for hydration gel manufacture.

[0072](Working example 5) Polly gamma-glutamic-acid molecular weight change of the strain by this invention which utilized Polly gamma-glutamic-acid decomposition activity measurement GPC of the strain by this invention, and followed culture time progress was investigated.

[0073]Cultivating the separation strain of this invention by GS solid medium, Polly gamma-glutamic acid was respectively refined by the aforementioned method on 1, 3, and the 5th at the time of progress, and a molecular weight and molecular weight distribution were investigated using GPC (Table 4).

[0074]

[Table 4]

***** (h)	*****	*****
0	1.204×10⁸	7.6
5	1.162×10⁸	7.8
9	1.037×10⁸	6.9

[0075]It turns out that the Polly gamma-glutamic acid compounded by the strain by this invention hardly changes an average molecular weight and molecular weight distribution even if culture time passes so that it may see in Table 4, therefore, strain bacillus by this invention Subtilis or [that a natto fermented soybeans and bean paste hot pot does not have Polly gamma-glutamic-acid decomposition activity] --- or it can be judged that there is not almost it.

[0076]

[Effect of the Invention]As it explains in detail by the above and being explained,Salt-tolerant strain bacillus which separated this invention from "******" (natto fermented soybeans and bean paste hot pot) which is a South Korean tradition beans fermented food Subtilis natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang, KCTC0697BP). And Polly gamma-glutamic acid which is edible, the water solubility, the negative ion nature, and the biodegradable polymer substance which are produced from said strain is provided. Bacillus of this invention Subtilis A natto fermented soybeans and bean paste hot pot (Bacillus subtilis var.chungkookjang) produces Polly gamma-glutamic acid with a larger molecular weight than Polly gamma-glutamic acid of the cell which a common genus Bacillus stock produces, The quantity of production is excellent again, and the Polly gamma-glutamic acid produced by the strain of this invention can be used for product development, such as a high-value-added cosmetics raw material, a desiccant, and biodegradable plastic material, useful by composition and chemical preparation of a derivative.

[0077]

[Layout Table]

<110>
Bioleaders Corporation
<120>
Bacillus subtilis var. chungkookjang Producing High Molecular Weight
Poly-gamma-glutamic Acid
<130> E01-009
<150>
KR2001-1481
<160> 2
<170>
Kopatent In 1.71
<210> 1
<211> 20
<212> DNA
<213>
Artificial Sequence
<220>
<223>
Single stranded oligonucleotide primer
<400> 1
agagtttgat
cctggctcag
<210> 2
<211> 20
<212> DNA
<213>
Artificial Sequence
<220>
<223> Single
stranded oligonucleotide primer
<400> 2

agaaggagg
tgatccagcc
[Amendment 3]
[Document to be Amended]Description
[Item(s) to be Amended]Brief explanation of the drawings
[Method of Amendment]Change
[Proposed Amendment]
[Brief Description of the Drawings]
[Drawing 1]It is a figure showing the cell wall by various intracellular enzymes, and the constituent synthetic pathway of Polly gamma-glutamic acid.
[Drawing 2]Bacillus of this invention Subtilis A natto fermented soybeans and bean paste hot pot and bacillus Subtilis It is a graph which compares the sodium chloride tolerance of fermented soybeans.
[Drawing 3]Bacillus of this invention Subtilis It is a gel electrical-and-electric-equipment **** photograph which shows the plasmid existence propriety of a natto fermented soybeans and bean paste hot pot and other comparison strains. M is 1kb rudder marker and A is a bacillus. As for Subtilis 168 and B, the strain by this invention and C are bacilli. Subtilis fermented-soybeans IFO3336 is expressed.
[Drawing 4]Bacillus of this invention strain based on 16S rDNA base sequence Subtilis It is a distribution diagram of a natto fermented soybeans and bean paste hot pot.
[Drawing 5]Bacillus of this invention Subtilis It is a concentration gradient SDS-PAGE gel electrical-and-electric-equipment **** photograph of the Polly gamma-glutamic acid produced by the natto fermented soybeans and bean paste hot pot and other comparison strains. M is a standard protein marker and 1 is a bacillus. Subtilis 168 and 2 is a bacillus. Subtilis fermented-soybeans IFO3336 and 3 expressed the strain by this invention.
[Drawing 6]Bacillus of this invention Subtilis It is the figure which expressed ly the Polly gamma-glutamic-acid biosynthetic path of the natto fermented soybeans and bean paste hot pot. 1 -- glutamine; -- the 2-oxo guru TAREDO amino mutase and 2 -- a glutamine synthetase. 3 -- L-glutamic acid; -- as for the pyruvic acid amino mutase and 4, the D-amino acid amino mutase and 6 are Polly gamma-glutamate synthesis enzymes alanine racemase and 5, and TCA expresses a tricarboxylic acid cycle.
[Drawing 7]bacillus of this invention Subtilis the gel chromatograph result of the Polly gamma-glutamic acid which the natto fermented soybeans and bean paste hot pot produced -- a table -- the bottom is a graph.

[Translation done.]

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(54) 감마-폴리글루탐산 생산용 배지

요약

본 발명은 탄소원으로 글루코오스 60-110 g/l을 함유하는, 미생물로부터 γ-폴리글루탐산(γ-PGA)을 생산하기 위한 배지에 관한 것이다. 본 발명의 배지를 이용하는 경우, 보다 저렴하고 높은 수율로 γ-폴리글루탐산을 생산할 수 있다.

명세서

발명의 상세한 설명

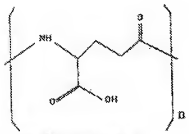
발명의 목적

발명이 속하는 기술분야 및 그 분야의 종래기술

본 발명은 γ-폴리글루탐산(γ-PGA) 생산용 배지에 관한 것이다.

γ-PGA의 구조는 다음과 같이 폴리아미드 구조를 갖고 있어 일종의 폴리펩타이드이나, α-카르복시기대신에 γ-카르복시기가 펩타이드 결합 형성에 관여한다는 점에서 일반적인 폴리펩타이드와는 다르다.

화학식 1



γ-폴리글루탐산(γ-PGA)의 구조

γ-PGA는 물질량이 수십만에서 몇백만 정도이며, 수용성이고 음이온을 띠고 있으며, 식용이 가능하다. 특히 이 화합물은 생분해성을 지니고 있어서, 약품이나 식품의 부형제, 포장재, 접착제 등으로 이용될 가능성이 높다.

γ-PGA는 미생물에 의해 생산되는데, Bacillus licheniformis는 세포외로 감마-폴리글루탐산(γ-PGA)를 생산한다고 알려져 있다.

Leonard, C. G., R. D. Housewright, and C. B. Thorne, 1958. Journal of Bacteriology, Vol 75,

pp.499-503에 따르면, Bacillus licheniformis를 배양하여 γ-PGA를 대량으로 생산하는 방법을 개시한다. 이 논문에서는 탄소원은 상대적으로 가격이 비싼 L-글루탐산, 시트르산과 글리세롤을 사용하였으며, 탄소원의 농도가 매우 높아, 이들 원료를 사용하면 γ-PGA에 생산원가가 높을 것으로 예측된다.

발명이 이루고자 하는 기술적 과제

본 발명에서는 γ-PGA의 생산단가를 낮출 수 있고 또한 γ-PGA를 높은 수율로 생산할 수 있는 배지를 제공한다.

본 발명에서는 가격이 비싼 탄소원 대신에 포도당을 주요 원료 탄소원으로 사용함으로써 γ-PGA의 생산단가를 낮출 수 있고 또한 γ-PGA를 높은 수율로 생산할 수 있다.

발명의 구성 및 작용

본 발명에 따른 배지는 탄소원으로 글루코오스를 80-110 g/l를 함유한다. L-글루탐산과 시트르산은 각각 0.5 g/l 이하로 함유한다.

질소원을 보충하기 위하여 NH_4Cl의 양을 보다 많이 함유한다.

기타 염류로는 미생물 배양 분야에서 널리 사용되는 각종 염류, 예를 들어 NH_4Cl, K_2HPO_4, KH_2PO_4, Na_2HPO_4 등이 사용될 수 있으며 그 종류는 제한되지 않는다. 또한 염류의 사용량 역시 미생물의 종류에 따라 적당하게 조절될 것이다.

이하 실시예를 통해 본 발명을 더욱 자세하 설명하고자 하나, 본 발명의 범위는 이들 실시예에 의해 제한되지 않는다.

[실시예 1]

실험에 사용된 배지의 성분은 표 1에 기재된 바와 같다.

[표 1]

Bacillus licheniformis에 의한 γ-PGA 생산용 배지 조성

구성성분	함량(gram/liter)								
	배지A	배지1	배지2	배지3	배지4	배지5	배지6	배지7	배지8
글루코오스	0.0	100	90	80	70	50	90	70	50
L-글루탐산	20.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
시트르산	12.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
글리세롤	80.0	0.0	0.0	0.0	0.0	0.0	10.0	30.0	50.0
NH_4Cl	7.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
K_2HPO_4	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KH_2PO_4	0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Na_2HPO_4 $12H_2O$	0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$MgSO_4$ $7H_2O$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$FeCl_3.6H_2O$	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
$CaCl_2.2H_2O$	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
$MnSO_4.H_2O$	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104	0.104

표 1에 따라 액체 배지를 만들었다. 포도당 용액은 여과하여 제균하였고, 염류의 침전 형성을 방지하기 위하여 $MgSO_4.7H_2O$, $FeCl_3.6H_2O$, CaCl

2 $2H_2O$와 $MnSO_4.H_2O$는 별도로 멸균한 후 배지에 첨가하였다.

3. 항균조에 배지 1L를 넣고, 냉동보존 중인 균주 배양액 1 ml을 가하여 배양을 시작하였다. 균주 배양 온도는 37℃, pH 6.5-6.8을 유지하였으며, 일정한 pH를 유지하기 위하여 2N HCl과 2N NaOH를 사용하였다. 균체 증식과 γ-PGA의 생산으로 인해 배양액의 점도가 증가하고 또한 신소 요구량이 증가하므로, 발효조의 교반속도는 50 rpm에서 500 rpm까지, 통기량은 0.5LPM에서 시작하여 2.0LPM까지 점진적으로 증가시켰다.

배지 A는 대조구로, 앞에 기재된 Leonard, C. G., R. O. Housewright, and C. E. Thorne, 1958. Journal

of Bacteriology, Vol. 76, pp.499-5030에 기재된 배지 조성에 따라 제조하였다.

배양중에 발효액을 5 ml씩 취하여 균체 농도의 변화와 γ-PGA 농도 변화를 관찰하였다. 균체 농도는 660 nm에서의 흡광도로 측정하였으며, γ-PGA 농도는 HPLC로 측정하였다. 발효 시간에 따른 γ-PGA의 농도변화를 측정한 결과를 표 2로 나타낸다.

[표 2]

발효시간에 따른 γ-PGA의 농도변화

발효시간(hour)		0	12	24	36	48	60	72	84	96
γ-PGA 농도 (g/l)	배지A	0.0	0.0	1.5	4.0	5.4	8.2	9.8	10.3	12.5
	배지1	0.0	0.5	2.5	4.8	11.2	12.0	12.5	12.8	13.0
	배지2	0.0	0.5	2.5	4.8	11.2	12.0	12.5	12.8	13.0
	배지3	0.0	0.5	2.5	4.8	11.2	12.0	12.5	12.8	13.0
	배지4	0.0	0.5	2.5	4.5	5.8	6.6	7.0	7.1	7.1
	배지5	0.0	0.5	2.5	4.0	4.2	4.3	4.4	4.5	4.5
	배지6	0.0	0.5	2.5	4.7	11.0	12.0	12.2	12.5	12.9
	배지7	0.0	0.5	2.5	4.7	10.0	10.8	11.4	11.8	12.5
	배지8	0.0	0.5	2.5	4.7	9.0	10.0	11.0	11.5	12.4

발명의 효과

주요 탄소원으로 글리세롤, L-글루탐산 또는 시트르산 대신에 글루코오스를 주원료로 사용하여 Bacillus licheniformis를 배양하면 보다 저렴하고 높은 수율로 γ-폴리글루탐산을 생산할 수 있다.

(57) 청구의 범위

청구항 1

바실러스 리케니포미스(Bacillus licheniformis)의 γ-폴리글루탐산(PGA) 생산용 배지에 있어서, 시트르산(0-0.5 g/l) 및 L-글루탐산(0-0.5 g/l)을 함유하고, 탄소원으로는 글루코스 80-110 g/l 만을 함유하는 것을 특징으로 하는 γ-폴리글루탐산(PGA) 생산용 배지